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CLXXV. "DIET 4" FOR BREEDING RATS FOR WORK ON VITAMIN A.

By SKÚLI VILHJÁLMUR GUDJÓNSSON.

Partly from the University Institute of Hygiene, Copenhagen.
(Professor L. S. Fridericia.)

(Received July 14th, 1930.)

The technique concerning breeding rats is described in detail in a previous publication [Gudjónsson, 1930]. As demonstrated there it was found that in several of their functions the rats were subject to seasonal variations. These variations appeared to have influence on the fore-period of the experimental rat, and their effect could be traced in the after-period, where they may be of decisive significance. The natural consequence of these findings was to try to improve the technique so that these sources of error were diminished or avoided. It did not seem unlikely that variations in the vitamin A content of the food given to the breeding rat might be one of the reasons for the variations in the experimental rats.

"Diet 4" for breeding rats.

It was therefore attempted to construct a diet for the breeding animals which should be adequate for the normal well-being of the animals, should vary as little as possible in its vitamin A content in the different seasons, and should contain the smallest adequate amount of this substance. Regardless of all theories I have composed the following diet for breeding rats:

Skimmed-milk powder	30 %
Rice flour	40 %
Autolysed yeast	15 %
Hardened coconut oil and shark-liver oil ...	15 %

The skimmed-milk powder was made from pasteurised milk. It contained only about 1 % fat, and should therefore contain only a minimum of vitamin A, or be entirely free of this substance. I also reckoned on it being poor in vitamin B. There is no need to take vitamin C into account. The rice flour is made from polished rice. The top yeast was autolysed at 40° and used without drying. The coconut oil contained 0·3 % shark-liver oil.

The intention was to give the rats a limited supply of vitamin A in a food mixture that would be adequate in other respects, so that rats could be bred which at the age of 30 days were not provided with different or much too great reserves of vitamin A. Their fore-period would then be shorter and more uniform than has been the case in previous experiments. By this means, the breeding of the animals was really made a part of the test system; the fore-

period was not only extended back to the birth of the rat but actually made to cover its descent. In addition, the stock of breeding animals that was raised on this diet was derived from only a few individuals.

In this way I have succeeded in creating a breeding stock that produces experimental material of very uniform quality. The diet proved to be perfect in every respect and did not call for anything in addition but water. The rats ate it well. The young rats throve on it and grew to normal size; there was never any sign of deficiency disease. This diet has been given to all the breeding animals for three years; now the 10th generation is thriving on it.

Some provisional results with this diet have already been given [Gudjónsson, 1930]. Since then a considerable number of experiments have been carried out with young rats whose parents had been fed on "Diet 4." The following is a summary of the results.

Fertility of rats on "Diet 4."

On this diet the fertility is somewhat less than it was on the old diet. Formerly there were about 50 litters a month per 100 females; on "Diet 4" about 40. A contributory cause of this fall is undoubtedly to be sought in the fact that at one time—about 1 year ago—several of the stock rats were getting too old.

Fig. 1 gives the curve for the number of litters per 100 females during the year (number of litters 312, mothers per month 67).

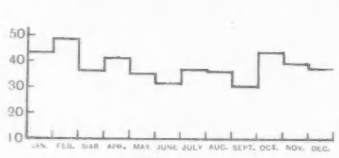

Fig. 1.

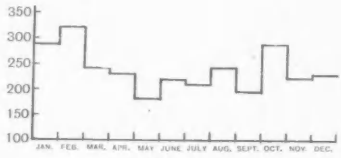

Fig. 2.

Fig. 1. Number of litters per 100 females on "Diet 4" in a breeding stock of about 40–50 females.
Fig. 2. Number of young per 100 females on "Diet 4" in a breeding stock of about 40–50 females.

Fig. 2 shows the number of young per 100 females during the year.

Fig. 3 shows the curve for litter size throughout the year. Here the variations are much smaller than among the rats on the old diet.

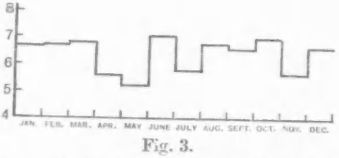

Fig. 3.

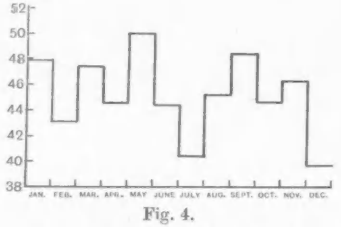

Fig. 4.

Fig. 3. Size of litters cast by females on "Diet 4" in a breeding stock of about 40–50 females.
Fig. 4. Average weight of 30 days old rats of "Diet 4" stock, tabulated according to month of birth. Number of rats about 1000.

"DIET 4" AND THE FORE-PERIOD.

(The fore-period means the period during which young rats get vitamin A-free diet until they begin to show typical symptoms of avitaminosis A.)

Investigation was also made into the average weight of the young rats at the age of 30 days, the length of the fore-period and the increase in weight during the fore-period in the different seasons.

Fig. 4 gives the average weight curve throughout the year for 30 days old rats. The observation period is 3 years. Generally the animals now weigh more than they did before on the old diet. This speaks for the quality of the diet.

Fig. 5 shows the average length of the fore-period.

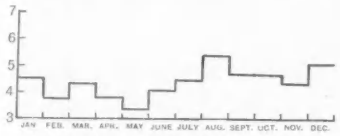

Fig. 5.

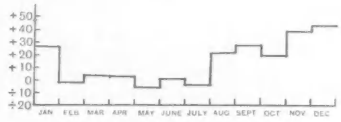

Fig. 6.

Fig. 5. Average length of fore-period in weeks among about 900 rats on vitamin A-free diet. All the rats came from mothers on "Diet 4."

Fig. 6. Average weight variation in the whole fore-period (figures along the ordinate), calculated in percentage of the initial weight, among about 900 rats on vitamin A-free diet. All the rats come from mothers on "Diet 4."

Fig. 6 gives the curve for the average weight variation throughout the fore-period. It is much flatter than the corresponding curve in the previous communication relating to breeding rats on ordinary diet. This and the last curve deviate markedly in a favourable direction from corresponding curves in the afore-mentioned paper, section 1. Here the individual variations in the rats are quite minimal; they were wide in the material studied before. The curve seems to show a tendency to greater increase in weight during the fore-period in the months August to January than in the spring and early summer months.

Here the average length of the fore-period for the whole year is about 4·4 weeks as against 8 weeks for animals on the old diet. Here the longest fore-period was 7·5 weeks; in the other animals more than 12·5 weeks. One would probably advance yet further towards an improved and uniform animal material by reducing all the litters to a certain number of young, 6, for instance, as is done by Macy, Outhouse, Long and Graham [1927].

With the breeding stock, diet and breeding technique employed for this material there is attained what was demonstrated in the previous paper to be the most essential object, *viz.* the production of an animal material for vitamin A tests in which all the rats have a favourable and uniform fore-period. This alone makes the results of the tests reliable.

In addition, the shortening of the fore-period is a great advantage as far as time, work and expense are concerned.

On this diet, all the 30 days old rats have probably about the same reserves of vitamin A. It does away with the necessity in Zilva and Miura's method [1921] for bringing the rats to a clinically manifest stage of avitaminosis A. It is even probable that these rats may be used for vitamin A tests as soon as they are 30 days old; and this will perhaps turn out to be the golden mean between the curative method and the preventive method. It may be, as suggested in the previous paper, that it will be possible to estimate the vitamin A content of a substance by the weight curve for the rats before they begin to fall off in normal weight-increase, the length of time being measured during which the reserves of the rat plus the added substance can keep up the normal weight-increase. It might then be possible to use some other standard instead of the weight—for instance, the time it takes for definite symptoms to develop, etc.

SUMMARY.

1. A description of "Diet 4" for rats kept for breeding purposes is given.
2. Seasonal variations, which are present in rats kept for breeding purposes and experimental rats fed on uncontrolled diet, have almost disappeared in rats fed on "Diet 4."
3. The rats breed well on this diet. The young rats are heavier than those bred on other diets and they are more suitable for vitamin A experiments. Experimental animal material of uniform and favourable quality for vitamin A experiments can be produced on "Diet 4", thereby ensuring that the assay of vitamin A by animal experiments becomes a matter of much greater certainty than hitherto.

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Macy, Outhouse, Long and Graham (1927). *J. Biol. Chem.* **73**, 153.
Zilva and Miura (1921). *Biochem. J.* **15**, 654.

CLXXVI. THE INFLUENCE OF FOODSTUFFS ON THE ACID-BASE BALANCE OF CATTLE URINE.

By FREDERICK JOHN WARTH and NARAYANA KRISHNA AYYAR.

From the Animal Nutrition Section, Imperial Department of Agriculture in India, Bangalore.

(*Received September 2nd, 1930.*)

Observations on the reaction of cattle urine are not met with very frequently in the literature. Hart and co-workers [1917], in their classical experiments on feeding with products from a single plant, observed that urine from animals fed with wheat straw was acid, while diets of oats and maize products led to alkaline urines. Carpenter [1927], studying the urine of fasting steers, pointed out that the normal herbivorous alkaline reaction became acid during fasting. A few p_H values are recorded, but as far as we are aware a fuller study of the acid-base balance has not been undertaken. The question came into prominence at Bangalore when feeding experiments were undertaken with rice straw. The urine from this fodder was found to be exceptionally large in volume, highly alkaline and extensively buffered with carbon dioxide. A suitable analytical procedure was then worked out, and with this we have studied the urines from a number of typical foodstuffs.

Methods.

The main determination was a potentiometric titration of CO_2-free urine prepared from the 24-hour sample. The double quinhydrone electrode was employed for the titration, the arrangement being on the lines described by Meeker and Oser [1926] and Morgulis and Hamsa [1927]. One modification introduced by us may be mentioned. To obtain accurate readings for both low and high values we used two comparison liquids, 0·1 *N* HCl for the low range and a buffer solution of p_H 2·2 for the high range. Both comparison liquids were connected by salt bridges to the titrating vessel and the change of buffer could be effected instantly by turning a tap and a switch. The voltmeter was standardised by means of buffer solutions of known p_H, but in actual practice we worked with empirical calibrations made by titrating mixtures of HCl and hippuric acid in the first place and then by titrating urine to which known amounts of one or other or both of these acids had been added. It should be mentioned here that in titrating cattle urines we have found no trace of discontinuity in the titration curve at the point where mineral acids are neutralised.

Tauffel and Wagner [1927] record a similar experience in titrating gastric juice in which they found "no marked discontinuity." Calibration must be resorted to under such circumstances. With our calibration we were able to measure added acids accurately and to distinguish mineral acids from organic acids clearly. Morgulis and Hamsa [1927] have shown that urine of the horse, the dog and man had very appreciable buffering values between p_H 7·4 and 8·0. We have observed the same effect to a less marked extent with cattle urines. This point is illustrated in the accompanying typical titration graphs obtained by us. It should be stated, however, that the data we give later all refer to titrations to the neutral point only.

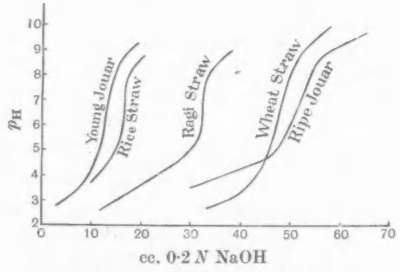

Fig. 1. Typical titration graphs of urines.

The preparation of CO_2-free urine for this titration was attempted in various ways. The procedure finally adopted was as follows. 50 cc. of urine are measured into an aspirating vessel, acidified with 10 cc. of 2·0 *N* HCl and aspirated. During the aspiration hippuric acid separates out, part of it settling, part becoming attached to the vessel and aspirating tube. The urine, when free of CO_2, is transferred to a 100 cc. flask. The aspirating apparatus is treated with 10 cc. of 2·0 *N* NaOH to dissolve the hippuric acid. This alkali is added to the urine, which is then made up to 100 cc. Thus 50 cc. of urine are deprived of CO_2 and made to 100 cc. with no other change than the addition of 10 cc. 2·0 *M* NaCl.

An aliquot portion of this liquid is measured into the titrating vessel, made decidedly acid with a measured amount of 0·2 *N* HCl and titrated in the usual manner. The amounts of mineral acid and organic acid present in the urine are obtained from the plotted data of this titration.

In addition to the potentiometric titration, chlorides, sulphates, ammonia and hippuric acid are determined in separate aliquots of the original urine sample. P_2O_5 was not determined because there is practically no inorganic P in these urines. The total P, of which the inorganic P forms a small fraction, is itself generally quite negligible in amount. Finally all bases and acids are expressed in cc. of 0·2 *N* per 25 cc. urine.

Calculation. The process of calculation will be made clear by the following example. 50 cc. of urine were deprived of CO_2 and brought to 100 cc. 50 cc. of this liquid, after acidification with 55·0 cc. of 0·2 *N* HCl, required 4·8 cc. alkali

to neutralise mineral acids and a further 17·0 cc. to neutralise organic acids. As 55 cc. HCl had been added to the liquid before titration, it is evident that the urine is strongly alkaline. From the analytical data the total alkalis and acids are calculated thus:

	cc. 0·2 *N*
Total alkalis	
Acid added to urine	55·0
Alkali required to neutralise mineral acids	4·8
Hence free alkali in urine (1)	50·2
Alkali present as chlorides (2)	15·2
„ as sulphates (2)	1·0
Total alkalis	66·4
Ammonia (3)	1·0
Therefore fixed alkalis	65·4
Total acids	
Organic acids by potentiometric titration	17·0
Chlorides (2)	15·2
Sulphates (2)	1·0
Total acids	33·2

(1) This alkali is present in the urine mainly as salts of organic acids, together with varying amounts of bicarbonate.

(2) Determined separately. The alkali chlorides and sulphates are not measured in the titration process.

(3) Determined separately.

This indirect process of estimating the total alkalis has been checked repeatedly by parallel gravimetric analysis and found to give satisfactory results. The data obtained in the last four tests are given in the accompanying table.

Table I. *Direct and indirect methods of determining alkalis.*

Alkalis present in 25 cc. urine (expressed in cc. 0·2 *N* alkali).

		Calf No. 832	Calf No. 840	Bullock I	Bullock II
Gravimetric determination	CaO	4·50	2·70	2·20	1·44
	MgO	12·35	10·44	6·62	8·53
	Alkali	50·25	45·62	49·50	51·25
	Total	67·10	58·76	58·32	61·22
Indirect determination	Titration	37·7	37·0	32·0	34·5
	Cl	27·4	18·9	25·0	25·4
	SO_4	2·6	3·2	2·7	3·4
	Total	67·7	59·1	59·7	63·3

The agreement between the indirect determination and the figures obtained by gravimetric analysis is amply sufficient for our purposes. The failure of Morgulis and Hamsa [1927] to obtain such agreement may be due to the fact that these authors estimated organic acids by the Van Slyke and Palmer colorimetric method [1920], which, though excellent for its intended purpose, is apparently not quite accurate enough to form a basis for the alkali calculation.

The total bases exist as salts of mineral acids, salts of organic acids and carbonates. Knowing the total bases, the mineral acids and the combined CO_2,

the organic acids should be obtainable by difference. Table II shows the close agreement between these two methods.

Table II. *Direct and indirect methods of determining organic acids.*

	Calf No. 832	Calf No. 840	Bullock I	Bullock II
Total bases (by titration)	58·1	59·2	59·7	63·3
Chlorides, sulphates and combined CO_2				
Bases present as inorganic salts	30·6	30·0	42·0	44·8
Bases present as organic salts (by difference)	27·5	29·2	17·7	18·5
Bases present as organic salts (by titrimetric method)	25·9	28·5	16·8	18·3

The CO_2 content and the p_H can only be determined on fresh urine. We found it desirable also to re-determine the ammonia on fresh samples. For these tests the urine was dealt with immediately it had been voided. Generally 4 to 6 samples were tested and the mean figure was taken to represent the 24-hour average.

It must be understood therefore that the CO_2, the p_H and the re-determined ammonia figures are not strictly comparable with the acid-base figures. This is unavoidable, but we believe each set gives a true picture of the urine.

Results.

We have examined the urine obtained from the following types of feeding: (*a*) green fodder and early cut hay as the sole food; (*b*) over-ripe hays fed with concentrate; and (*c*) cereal straws fed with concentrate. The urine was obtained from mature bullocks undergoing digestion trials and receiving maintenance rations. The urine when voided was put into a bottle containing toluene and was preserved in an ice-box overnight. The acid-base balance was determined on 3 successive days on the 24-hour sample and the average of the three determinations recorded. The alkali figures were obtained by the indirect process outlined above. After the acid-base balance had been determined 2 days were devoted to the examination of fresh urine samples. The results are set out in Table III in which the following points may be noted.

Carbon dioxide is the most variable constituent, its concentration ranging from 0·6 to 43·4. Further, the close relationship between CO_2 concentration and "excess of alkali" is striking, showing that the buffer effect of CO_2 is a prominent feature in many of these urines. Hippuric acid and chlorides also vary to a considerable extent, the former ranging between 6·7 and 35·9, the latter between 10·0 and 37·3. Total bases, largely potash, are relatively constant, showing that the urine volume is regulated largely by the amount of potash in the food. Owing to the absence of phosphoric acid these urines are buffered by organic acids and CO_2. It may be noted that on the average two-thirds of the total organic acid consists of hippuric acid. This acid is therefore of prime significance in the acid-base balance. The p_H of our urine samples ranges between 5·7 and 8·5 according to the nature of the food; the p_H values of the corresponding faeces range between 7·2 and 8·2.

Table III. *Acid-base balance of cattle urine.*

Acids and bases are expressed as cc. 0·2 *N* in 25 cc. urine.

	Green fodders							Over-ripe grasses						Cereal straws				
	Prime Jouar	Jouar (young green)	Jouar (young green silage)	Rhodes grass	Dhub hay	Dhub grass	Guinea grass	Tornagallu hay	Aurangabad hay	Ripe Jouar	Bolarum hay	Ripe Jouar silage	Ripe Jouar	Rice straw	Millet straw	Oats straw	Ragi straw	Wheat straw silage
Total bases	69·0	68·2	59·2	68·9	57·3	47·6	53·2	51·5	62·6	66·7	75·5	64·0	74·8	61·6	69·1	63·6	64·3	43·7
Chlorides	10·7	15·8	9·2	24·5	19·1	16·7	37·3	14·7	24·3	33·1	22·9	33·3	32·5	10·0	12·8	22·0	25·3	24·9
Sulphates	1·0	1·0	0·7	4·1	5·3	6·5	0·7	3·1	0·6	1·6	1·0	—	3·6	0·3	1·3	2·3	2·5	1·8
Hippuric acid	19·2	15·1	18·3	15·4	11·3	8·8	6·7	23·4	26·2	22·2	35·9	20·8	30·8	9·3	13·4	9·3	12·5	9·2
Other organic acids	7·5	6·3	9·0		6·9	5·6	2·7	9·2	10·4	10·2	13·5	7·5	8·3	6·3	7·8	5·2	7·5	6·0
Total acids	38·4	38·2	37·2	44·0	42·6	37·6	47·4	50·4	61·5	67·1	73·3	61·6	75·2	25·9	35·3	38·8	47·8	41·9
Excess of alkali	30·6	30·0	22·0	24·9	14·7	10·0	5·8	1·1	1·1	−0·4	2·2	2·4	−0·4	35·7	33·8	24·8	16·5	1·8
CO_2	43·0	37·0	35·1	30·2	26·0	24·5	5·8	4·3	2·9	1·3	1·0	0·8	0·6	51·6	43·8	36·9	25·3	2·5
Ammonia	0·3	0·3	—	—	0·5	0·5	—	0·7	0·8	—	6·9	—	—	0·2	0·5	0·4	0·2	—
p_H of urine	8·2	8·2	8·2	8·5	8·2	8·3	8·2	7·5	6·5	5·7	6·1	6·7	6·0	8·3	8·3	8·3	8·1	6·7
p_H of faeces	7·6	7·6	—	—	7·8	7·6	—	7·9	7·85	—	7·2	—	—	8·0	8·2	7·5	7·8	—
Vol. of urine in litres per day	6·73	5·80	4·81	5·20	4·39	5·56	6·97	2·93	2·75	3·42	1·55	4·05	3·48	5·93	4·84	6·95	4·10	5·39
Ratio Hippuric acid/ total organic acid	0·719	0·706	0·670	—	0·621	0·611	0·713	0·718	0·716	0·685	0·727	0·735	0·788	0·596	0·632	0·641	0·622	0·605

Ammonia in fresh urine was not determined in all cases. Every urine tested, even the most alkaline, gave small traces of ammonia. The one acid urine which was tested gave a much higher figure. The general characteristics of the urines from the three types of fodder are as follows. (*a*) Urine of green fodders: excess of alkali, buffering with CO_2 and high p_H. (*b*) Urine from over-ripe grasses: no excess of alkali, very little CO_2 and low p_H. (*c*) Urine from cereal straws: excess of alkali, buffering with CO_2 and high p_H. Wheat straw is an exception in its class. The acidity of urine from wheat straw has however been observed by Hart and co-workers [1917]. Our result is therefore not due to peculiar circumstances of soil or climate.

A negative chloride balance was observed in animals fed on Bolarum and Tornagallu hays and on rice and millet straws, indicating that these fodders are deficient in chloride.

The general similarity of urines from green fodders and from cereal straws is remarkable and perhaps significant. The peculiar urines from over-ripe hays stand out in sharp contrast to the alkaline urines from other fodders. This peculiarity may have considerable significance in India, where, for several months in the year, over-ripe hay forms the staple food of the grazing herds. It is not suggested that acidity of urine must necessarily result from all over-ripe hays. No doubt soil and climate modify the result. We have proof however in one case that ripening of the crop is a factor in altering the urine reaction. A uniform crop of jouar on the Bangalore farm was harvested at three stages.

The early cut gave an alkaline urine, the second cut neutral urine, and the third cut a highly acid urine. We have evidence also that the nature of the season may greatly modify results. The above experiment was repeated during the following season which happened to be abnormal. Owing to late rains growth persisted much later and the final crop did not produce acid urine.

SUMMARY.

1. A method is described for the determination of the acid-base balance of cattle urine.

2. The urines from three types of fodders were examined by this method. It was found that the urines from green fodders and cereal straws were alike in containing excess of alkali and large amounts of CO_2. In contrast to the above, the urines from over-ripe hays contained very little CO_2 and were neutral or acid. The urine of wheat straw is acid and forms an exception in its class.

3. It was found that progressive ripening of a fodder tends to increase the acidity of the urine. At the same time, it was observed that the climatic conditions may materially modify the effect of ripening.

4. These urines contain only traces of phosphoric acid. The buffering is effected by organic acids of which hippuric acid forms nearly two-thirds. This acid is therefore highly significant in the acid-base balance.

5. When the organic acids are deficient, CO_2 comes in as a subsidiary buffer. The amount of CO_2 present in the urine is found to increase regularly with the excess of alkali.

6. A subsidiary observation showed that some of these fodders are deficient in chlorides.

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CLXXVII. THE COLORIMETRIC DETERMINATION OF THE TYROSINE AND TRYPTOPHAN CONTENT OF VARIOUS CRUDE PROTEIN CONCENTRATES.

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In the course of investigations on poultry nutrition, it was desired to know the tyrosine and tryptophan content of the protein of buttermilk powder, fish meal, cod-liver meal, and two abattoir by-products, meat meal and tankage. A rapid and reliable method for the estimation of tyrosine and tryptophan in foodstuffs would be an extremely valuable asset, not only in regard to our particular problem, but in a multitude of nutritional investigations.

At the present time it is doubtful whether any of the known methods for determining the tyrosine, and particularly the tryptophan, content of purified proteins are absolutely reliable. Onslow [1924], summarising the values given for the tryptophan content of caseinogen by different investigators, has pointed out the great discrepancies in the results obtained. Though the colorimetric methods have sometimes given low values, and are open to the usual objections to colorimetric methods in general, they are the only methods which could be satisfactorily applied to the determination of tyrosine and tryptophan in a routine way, such as we contemplated.

It was hoped that after making as thorough a purification of these materials as was possible, results, of sufficient accuracy to compare the tyrosine and tryptophan content of one material with another, might be obtained by colorimetric methods. Fürth and Lieben [1921] and Ide [1921], using a colorimetric method based on Voisenet's reaction [1905], have determined the tryptophan content of many of the common foodstuffs. The unreliability of the Voisenet formaldehyde reaction has been shown by Hunter and Borsook [1923], who state that glycyltryptophan gives a far deeper and redder colour than pure tryptophan. This reaction is thus affected both as to hue and intensity, not only by the presence of tryptophan, but by the mode of combination of tryptophan in the protein molecule. Kretz [1922] in endeavouring to use this method for localising tryptophan in plant cells has concluded that it did not give accurate results because of the susceptibility of the reaction to many destructive influences. The colorimetric method of Folin and Ciocalteu [1927]

for determining tyrosine and tryptophan commended itself. The two determinations are combined into one procedure, which is reasonably rapid, and the accuracy has been subjected to considerable study in so far as its application to purified protein is concerned. Whether accurate determinations of the tyrosine and tryptophan content of these crude protein materials could be made by this method, or by other methods discussed in the text, remained to be determined.

Experimental.

Representative samples of these protein supplements were prepared for analysis as follows. They were ground in a Wiley mill to pass through a 100 mesh sieve and then dried *in vacuo* at 100° and 5 mm. for 24 hours. The dried samples were then extracted with sodium-dried ether in a large Soxhlet extractor until fat-free, which usually took about 48 hours. The residues were placed in 250 cc. centrifuge bottles and extracted with two volumes of absolute alcohol by shaking for 12 hours. After centrifuging, the alcohol was decanted and the extraction repeated three times as before. The residues were again dried in the vacuum oven and finally stored in tightly stoppered bottles. All subsequent weighing of samples for analysis was made by weighing the stoppered tube before and after the transfer. Total nitrogen was determined (Kjeldahl) and all subsequent determinations are expressed as % of the total crude protein (N × 6·25). The tyrosine and tryptophan determinations were first made by Folin and Ciocalteu's method. A sample of purified caseinogen on repeated analysis was consistently found to contain 6·62 % tyrosine and 1·37 % tryptophan. The values obtained by Folin and Ciocalteu for a highly purified caseinogen were 6·55 % tyrosine and 1·40 % tryptophan.

In each case a weighed quantity of the protein material representing the equivalent of 1 g. of crude protein was used in duplicate determinations. The alkali digests, excepting the caseinogen digest, were highly coloured and in the subsequent determinations of tyrosine it was found to be impossible to make accurate colour comparisons against the standard solution in the colorimeter. It was then decided to decolorise the hydrolysates by shaking with 1 g. of kaolin for 12 hours and centrifuging. By similar treatment of standard solutions of tyrosine and tryptophan it was determined that no loss of these amino-acids by absorption took place. While norite and animal charcoal made much better decolorising agents, a very considerable loss, particularly of tyrosine, was found to take place. The results of the determinations on the decolorised hydrolysates, expressed as % of the total crude protein (N × 6·25), were as follows:

	Tyrosine	Tryptophan
Meat meal	2·93	1·34
Tankage	3·02	0·67
Fish meal	2·61	1·30
Cod-liver meal	—	2·04
Buttermilk powder	5·46	1·80

With the phenol reagent (tryptophan) the colour comparison with the unknown and the standard was excellent in every case and duplicate determinations gave practically the same results. With Millon's reagent (tyrosine) the colour comparison was not perfect, the unknown having a yellowish red colour and being slightly cloudy, while the standard was bright red in colour and perfectly clear. The readings were made as carefully as possible but leave some doubt as to their accuracy. It was found impossible to determine the tyrosine content of the cod-liver meal protein by this procedure as, on boiling the tyrosine-containing solution with $HgSO_4$ and cooling, the solution became very cloudy. Careful regulation of the acidity of the solution at this point, varying the acidity in repeated determinations, or carefully avoiding undue delay in making the colour comparisons, did not prevent this fine grey cloudy precipitate from forming with the other hydrolysates. Making the solution (after heating with $HgSO_4$) up to volume with N H_2SO_4 instead of water slightly improved the readings, especially in the case of the fish meal hydrolysate.

Hanke [1928], in a criticism of Folin and Ciocalteu's method for determining tyrosine, maintains that tyrosine cannot be determined by this method when cystine is present. Cystine gives a precipitate and this precipitate contains tyrosine. He has proposed a method for determining tyrosine which consists in hydrolysing the protein with H_2SO_4 and then removing the H_2SO_4 with baryta. The tyrosine is precipitated, even from a mixture of amino-acids and such other substances as might be present in a protein hydrolysate, by boiling in acetic acid solution with mercuric acetate and treating the mixture with NaCl. The mercury is removed by H_2S and the tyrosine determined in the filtrate colorimetrically with Millon's reagent, as in the Folin and Ciocalteu procedure. More accurate determinations of tyrosine in these crude protein materials might be expected by actual precipitation of the tyrosine in the hydrolysate, rather than by a colorimetric determination in the presence of all possible interfering substances.

The tyrosine content of these proteins was next determined by Hanke's method with the following results:

	% tyrosine	
	Hanke's method	Folin and Ciocalteu's method
Caseinogen	6·59	6·62
Meat meal	2·35	2·93
Tankage	2·51	3·02
Cod-liver meal	3·60	—
Fish meal	2·64	2·61
Buttermilk powder	5·28	5·46

In every case perfect colorimeter readings were obtained by this method. Excepting in the fish meal proteins, the tyrosine determination by Folin and Ciocalteu's method was, in every case, higher than that obtained by Hanke's method. Higher values were almost invariably obtained by Hanke, using Folin and Ciocalteu's procedure on purified proteins.

The tyrosine content of meat meal, tankage, fish meal and cod-liver meal is

much lower than that of the milk proteins. Ingvaldsen [1929] reports for fish meals from different sources and processed at different temperatures a tyrosine content of the proteins (N × 6·25) varying from 3·85 to 4·43 % determined by Folin and Ciocalteu's method. He does not report encountering any difficulties in the application of this method to fish meal hydrolysates. With the possible exception of tankage, these protein materials appear to be well supplied with tryptophan when compared with the value for caseinogen. The question still remains as to whether these figures represent the actual tryptophan content of these materials.

Hanke [1928] states that "a crude protein hydrolysate, either acid or alkaline, appears to contain something other than tyrosine or tryptophan that reacts with both the phenol reagent and Millon reagent of Folin and Ciocalteu. This may be one substance or there may be several; but whatever it is, it does not react in an identical manner with both reagents." In criticising the use of the phenol reagent for determining tryptophan, Kraus [1925] has pointed out that indole and skatole give a blue colour with the phenol reagent so that decomposition products of tryptophan would also be included. Indole and skatole, however, are extremely soluble in ether, and were they present in the original materials as the result of putrefaction they would have been removed in the preparation of these products for analysis. It is possible, however, that in the alkali-digestion of these crude protein materials considerable reduction of tryptophan may take place. Indole and skatole, according to Kraus, may be removed quantitatively from the hydrolysates by extraction with toluene.

The original decolorised hydrolysates were next extracted with toluene by continuous shaking for 12 hours, allowed to stand for 2 hours in separating funnels and then separated from the toluene. That tyrosine and tryptophan are not removed from solution by toluene was determined by a similar treatment of standard solutions. The tyrosine and tryptophan contents of the toluene-extracted hydrolysates were next determined by Folin and Ciocalteu's method with the following results:

	In original decolorised hydrolysate		After extraction of hydrolysate with toluene	
	Tryptophan	Tyrosine	Tryptophan	Tyrosine
Caseinogen	1·37	6·62	1·23	6·55
Meat meal	1·34	2·93	0·90	2·31
Tankage	0·67	3·02	0·59	2·92
Fish meal	1·30	2·61	1·17	1·83
Cod-liver meal	2·04	—	0·77	—
Buttermilk powder	1·80	5·46	1·51	5·40

A considerable reduction in the values for the tryptophan and tyrosine content in all cases was found. A reduction in the apparent tryptophan content of cod-liver meal of 1·27 % was very surprising. If the substances giving the reactions of tryptophan (*i.e.* precipitated in acid solution with $HgSO_4$ and giving a blue colour with the phenol reagent) but removable with toluene, are indole and skatole, it would seem that they must have been produced during the alkali-digestion of the protein material.

It was next decided to determine if tyrosine and tryptophan added to these protein materials could be recovered quantitatively in the alkali hydrolysates. Weighed quantities of Pfanstiehl C.P. tyrosine and tryptophan were added to weighed quantities of the prepared meat meal and fish meal as used in the previous determinations. The alkali-digestion and the subsequent determination of tyrosine and tryptophan were carried out as before. The results were as follows:

	Tyrosine		Tryptophan	
	Added (mg.)	Recovered (mg.)	Added (mg.)	Recovered (mg.)
Meat meal	45·0	40·3	4·0	3·37
Fish meal	40·1	44·0	6·4	7·0

It will be observed that in some cases more tyrosine and tryptophan was recovered than had been added. This can only be explained by the slight improvement in the readings obtained where tyrosine and tryptophan had been added. In these readings the colour of the unknown was more nearly proportional to the colour developed by the standard solution. The results, however, indicate that there was no appreciable destruction of added tyrosine or tryptophan during the alkali-digestion of these two crude protein materials. Results obtained after extraction of the meat meal and fish meal hydrolysate with toluene gave practically the same recovery of added tyrosine and tryptophan as recorded above. Evidently, only negligible amounts of decomposition products of tryptophan or tyrosine soluble in toluene were formed as the result of alkali-digestion.

The next endeavour was to determine what the substance (or substances) present in the alkali hydrolysates of these materials was, which gave the reactions of tryptophan but which could be extracted by toluene. The toluene extracts of all the hydrolysates gave a blue colour of varying intensity with the phenol reagent. The blue colour was particularly deep in the case of the toluene extract of the cod-liver meal hydrolysate, and least in the toluene extract of the caseinogen hydrolysate. Evidently the unknown substance was present in the greatest amount in the hydrolysate of cod-liver meal. That the original cod-liver meal contained no indole was determined by applying Bergeim's [Hawk and Bergeim, 1927] method for the quantitative determination of indole in faeces. Negative results were also obtained on the alkali hydrolysate of cod-liver meal.

The toluene extract of these hydrolysates gives Millon's reaction, which indicates that this unknown substance may be phenolic. Negative results were obtained for free and conjugated phenols by Tisdall's [1920] modification of the Folin and Denis method. Uric acid gives a blue colour with the phenol reagent; it is soluble in NaOH but is precipitated by H_2SO_4. However, sufficient remains in solution to give a fairly heavy precipitate with $HgSO_4$. While uric acid was found to be insoluble in toluene it may be that some other purine base, or some pyrimidine base, set free by decomposition of nucleoprotein is

contributing to the blue colour given by tryptophan. The actual purine content of the ox-liver as reported by Vogel [1911] is only 0·099 %.

While the colour given by tyrosine with the phenol reagent is a different shade of blue from that given by tryptophan, the colour given by indole is identical with that given by tryptophan. The comparative solubilities of indole did not indicate that, even if indole were present in the hydrolysates, it would increase the tryptophan reading. To confirm this 0·0833 g. indole was added to 0·9327 g. caseinogen and the determination of tryptophan carried out in the usual manner. A reading representing 2·46 % tryptophan was obtained while caseinogen contains 1·37 % tryptophan. The apparent tryptophan content of caseinogen was increased by 1·09 % by adding 8·9 % indole. The presence of indole in the hydrolysates would, undoubtedly, increase the tryptophan reading. The colour given by Millon's reagent, in determining tyrosine in presence of indole, was not rose-pink but a brownish yellow, and was somewhat typical of the colour given by the crude protein hydrolysates with Millon's reagent. Inorganic substances, such as ferrous iron or any sulphite, produce a deep blue colour with the phenol reagent. It would seem, as Gortner and Holm [1920] have pointed out, that any substance which is easily oxidised will react with the phenol reagent and produce a blue colour.

That some substance is present in these alkali hydrolysates which shows the reactions of tryptophan, but which evidently is not this amino-acid, has been reported by Hanke [1928] and by Kraus [1925], independently, and is confirmed by the above findings. The evidence does not point to this substance as being indole as Kraus has concluded. Until this unknown chromogenic substance has been identified, the results obtained after extraction of these crude protein hydrolysates with toluene would appear to represent the true tyrosine and tryptophan content of these protein materials.

An endeavour was made to determine the tryptophan content of these materials by the method of Komm [1926], based upon the reaction of tryptophan with *p*-dimethylaminobenzaldehyde. A value for caseinogen of 2·39 % tryptophan was obtained. While the tryptophan standard gave a uniform blue colour, the colour given by caseinogen was more of a purplish tinge. The colour comparison was not perfect and as a result the figure for caseinogen is probably too high. All the other determinations were a complete failure, owing to the deep brown colour produced on adding concentrated H_2SO_4 which prevented any possible colour comparison.

The tyrosine and tryptophan content of these crude protein materials as determined by enzymic hydrolysis.

A weighed quantity of the crude protein material (approximately the equivalent of 1 g. crude protein) was digested for 10 hours at 37° in 50 cc. 0·1 *N* HCl containing 0·2 % pepsin. The digest was neutralised with 5 cc. *N* NaOH and 5 cc. of a 6 % solution of trypsin in 0·1 *N* NaOH added. Digestion was then continued for 24 hours. Toluene was added as a preservative. The

enzyme activity was destroyed by heating on a water-bath for 5 minutes at 75°–80°. The digests were then acidified with H_2SO_4, diluted to 100 cc. and filtered. The addition of a small amount of kaolin greatly facilitated obtaining a clear filtrate. Tyrosine and tryptophan determinations were then made on an 8 cc. aliquot of the filtrate by Folin and Ciocalteu's procedure. Correction was made for the tyrosine and tryptophan content of the enzyme blank, obtained by adding known amounts of standard solutions of tyrosine and tryptophan to the blank pepsin-trypsin digest and so determining the tyrosine and tryptophan content of the latter by difference. The following results were obtained:

	Amino-N %	Tyrosine %	Tryptophan %
Caseinogen	8·31	7·59	1·54
Buttermilk powder	7·60	6·36	1·25*
Meat meal	4·97	3·02	0·95
Tankage	4·06	2·42	0·88
Cod-liver meal	2·25	—	0·77
Fish meal	5·43	2·92	1·38

* This solution was persistently cloudy and accurate colour comparison was extremely difficult. The result is, therefore, very doubtful.

A much higher reading was obtained for caseinogen by this method than was obtained by alkali-hydrolysis. The results obtained for meat meal were somewhat the same as before, while the tryptophan content of tankage was slightly higher. Fish meal would seem to contain considerably more tryptophan than meat meal and tankage. It was interesting to find that the tryptophan content of cod-liver meal, as determined by this method, was exactly the same as that obtained after extracting the alkali hydrolysate with toluene. This may have been simply a coincidence, but the fact that the results were very much of the same order suggests strongly that the unknown chromogenic substance present in the alkali hydrolysate of cod-liver meal in particular was formed during alkali-digestion.

Ragins [1928, 1] has recently reported the perfecting of her method for the determination of tryptophan by means of the vanillin-HCl method, so that it may be applied to proteins. She claims that this reaction for tryptophan is more sensitive than other colour reactions and that it is more specific. The method has been applied with evident success to the tryptic hydrolysates of highly purified proteins. An endeavour to determine the tryptophan content of these crude protein materials by this method and to compare the results with those obtained by Folin and Ciocalteu's method on the same hydrolysate was next made.

1 g. of the protein material and 0·25 g. U.S.P. trypsin were weighed into 100 cc. Erlenmeyer flasks containing 25 cc. 0·4 % Na_2CO_3. The solutions were preserved with toluene and incubated for 5 days at 37°. The enzyme was destroyed by heating at 75°–80° on the water-bath for 5 minutes. The digest was then acidified with 1 cc. 14 *N* H_2SO_4, diluted to 100 cc. and filtered. Tryptophan determinations were then made on an 8 cc. aliquot of the digest by Folin and Ciocalteu's method and on 3 cc. by the vanillin-HCl method.

	Tryptophan %		
	Phenol reagent	Vanillin-HCl	Remarks on colour with vanillin-HCl reagent
Caseinogen	1·25	1·30	Not identical with the standard but good colour comparison
Meat meal	0·52*	—	Reddish blue colour, impossible to match but qualitative indication that tryptophan was present
Tankage	—*	—	Red colour—no indication of blue colour
Cod-liver meal	1·21*	—	Deep brownish red colour
Fish meal	0·59*	—	Reddish blue colour as with meat meal digest
Buttermilk powder	1·87	1·73	Colour identical with that developed with caseinogen digest

* 2 cc. standard tryptophan solution (0·59 mg. tryptophan) were added to 8 cc. of the digest and tryptophan determined by difference. In the tankage digest the same amount of tryptophan was recovered as was added. The filtrate from the $HgSO_4$ precipitate in every case gave a negative test with the Hopkins-Cole reagent.

Tryptophan determinations by the phenol reagent in the tryptic digest of meat meal and fish meal were much lower than the previous results obtained by peptic-tryptic digestion. The preliminary digestion with pepsin resulted in a very considerable increase in the amount of tryptophan liberated. The negative result with the tankage digest, and more particularly the higher value obtained for cod-liver meal, would seem to be impossible to interpret.

Except in the case of the milk products, the results with the vanillin-HCl reagent were a uniform failure even when the determinations were repeated after decolorising the hydrolysates with kaolin. Some other substance present was found to develop a red colour with the reagent which made colorimeter readings impossible. The result for caseinogen is in fair agreement with that obtained by Ragins, *i.e.* 1·23 % tryptophan.

Waterman and Jones [1921, 1922] have found their determinations of the relative digestibility of proteins *in vitro* to correspond with protein digestibility trials *in vivo*. It was next decided to repeat these digestions by their method and to determine the tryptophan content.

0·5 g. of the protein material was digested for $1\frac{1}{2}$ hours at 37° in 50 cc. 0·1 *N* HCl containing 0·2 % pepsin[1]. The digest was neutralised with 5 cc. *N* NaOH and 5 cc. of a 6 % solution of trypsin[1] in 0·1 *N* NaOH added. Digestion was continued for $2\frac{1}{2}$ hours. The enzyme was destroyed by heating on a water-bath for 5 minutes at 75°–80°. The digest was then acidified with 1 cc. 14 *N* H_2SO_4, diluted to 100 cc. and filtered. A 3 cc. aliquot was taken for the amino-nitrogen determinations (Van Slyke) and a 5 cc. aliquot for the tryptophan determinations by the vanillin-HCl procedure. Tryptophan in concentrations of less than 0·2 mg. in a 5 cc. aliquot cannot be precipitated quantitatively under the conditions described. Therefore, 2 cc. of the standard tryptophan solution (the same amount as the standard) were added to each digest and tryptophan determined by difference, allowance being made for the tryptophan determined in the blank enzyme digest. The colours developed by the digests were much the same as already described; the interfering red colour making

[1] Products of the Digestive Ferments Co., Detroit, Mich.

accurate comparison with the standard impossible. It was found that by placing a blue-green (minus red) filter, which only admitted light of wave lengths 3800 Å.–5800 Å., between the colorimeter lamp and the Duboscq colorimeter excellent colorimeter readings could be obtained. The results were as follows:

	Amino-N %	Tryptophan %
Caseinogen	4·99	1·72
Buttermilk powder	3·18	2·95
Meat meal	3·96	1·78
Tankage	2·86	0·92
Fish meal	4·08	1·62

The results obtained were much higher than in all previous determinations but, comparatively, they fell into the same order as before. Preliminary digestion of these crude protein materials with pepsin, followed by trypsin, and limitation of the duration of digestion have given higher values for tryptophan liberated. Ragins [1928, 2] has found that treating the purified proteins which she has studied (caseinogen, edestin and squash seed-globulin) first with pepsin and then with trypsin, does not hasten or increase the liberation of tryptophan; also that only the enzymes in U.S.P. pancreatin liberate tryptophan from the proteins studied. Frankel [1916] found that proteins after having been acted upon by pepsin are much more readily digested by trypsin or erepsin. No definite interpretation can be placed on the results obtained with these crude protein materials until much more comprehensive experiments, determining the rate at which tryptophan is liberated with changing time of peptic and tryptic digestion, have been made. Excepting caseinogen and buttermilk powder, a considerable amount of these crude protein materials remains in suspension during enzyme digestion, so that accurate results cannot be obtained by removing aliquots as digestion proceeds.

Summary.

1. The tyrosine and tryptophan content of the proteins of buttermilk powder has been found to be much higher than that of the other crude protein materials investigated. No very appreciable difference in the tyrosine content of fish meal, cod-liver meal, meat meal and tankage was found.

2. Conflicting results as to the tryptophan content of the proteins of fish meal and meat meal, depending upon the method of determination, have been found. In general, the tryptophan content of fish meal was found to be higher than that of meat meal. The tryptophan content of tankage has invariably been found to be much lower than that of fish meal and meat meal.

3. The limitations in the methods described for determining tryptophan leave some doubt as to the actual tryptophan content of any of these materials, particularly in the case of the cod-liver meal proteins. The alkali digest of these crude protein materials contains some substance, or substances, precipitable by $HgSO_4$ and giving a blue colour with the phenol reagent which, unlike tryptophan, is soluble in toluene. This unknown chromogenic substance does

not appear to be indole as Kraus has concluded. The results obtained after extraction of the alkali hydrolysates with toluene would appear to represent the true tyrosine and tryptophan content of these protein concentrates.

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CLXXVIII. STUDIES IN EMBRYONIC MORTALITY IN THE CHICK.

I. THE EFFECT OF DIET UPON THE NITROGEN, AMINO-NITROGEN, TYROSINE, TRYPTOPHAN, CYSTINE AND IRON CONTENT OF THE PROTEINS AND ON THE TOTAL COPPER OF THE HEN'S EGG.

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While it is an undoubted fact that the successful hatching of a fertile egg is influenced by genetic inheritance and by the physical factors involved in the process of incubation itself, it has not been so definitely established that factors in the diet of the hen play an important part in embryonic mortality.

Our attention was drawn by Graham and Smith [1929] to the fact that possibly the solution of many of the problems of incubational mortality lay in the realms of physiological chemistry rather than in morphology. For some years past they have studied on an extremely comprehensive scale the influence of the diet of the parent fowl on egg production and on the hatchability of the eggs. Their results, of the incubation under standard conditions of over forty thousand eggs from hens of the same breeding and under controlled environmental conditions, show conclusively that the diet of the laying hen, particularly in regard to the supply of fat-soluble vitamins and the source of protein, has a profound influence both on the extent and the distribution of the mortality during the period of incubation. This dietary effect is pronounced apart from the apparent influence of individual constitutional vigour.

It is also apparent from their results that the influence of diet on hatchability is particularly pronounced during the months of February, March and April and not so apparent during May, June and July. It is to be anticipated that a deficient diet would have its greatest effect on hatchability during the mid-winter months when constitutional vigour is probably at its lowest ebb.

One of the most important generalisations that can be made as the result of modern investigations in avian nutrition is that it is not possible to secure a diet composed entirely of cereal grains and grain by-products which will promote normal growth and reproduction over any considerable period, and

that it is necessary to supplement with suitable proteins. The results of Graham and Smith show that so far as embryonic mortality is concerned it is not a matter of indifference whether the vegetable basal ration be supplemented with equal amounts of crude protein from either buttermilk powder, fish meal, cod-liver meal, meat meal or tankage.

In the winter months the mortality during incubation was greatest where meat meal, and particularly tankage, was fed as the only protein supplement. In the case of the latter the addition of cod-liver oil made very little difference. The least mortality was found where buttermilk powder was fed, especially when cod-liver oil or ultra-violet irradiation was given in addition. The feeding of the two abattoir by-products, meat meal and tankage, was found particularly to increase the embryonic mortality during the early period of incubation (1 to 6 days), this effect being most pronounced in the winter hatches. It was found that a considerable number of these embryos at three to four days development were in an anaemic condition, this being particularly prevalent in the embryos from the tankage diet.

It would appear that the numerous factors affecting the hatchability of hen's eggs have a far greater significance than is indicated by the meagre literature dealing with the problem. The investigations reported in this paper were initiated to determine whether any relationship existed between the chemical composition and the hatchability of eggs laid by hens on diets differing only in the source of protein concentrate fed. It was hoped that some light might be thrown on the dietary factors responsible for incubational mortality.

Experimental.

In the experiments conducted by Graham and Smith [1929] mature pullets, which had been raised under range conditions and which had previously been receiving the regular plant diet, were placed on the experimental diets. The influence of a diet on hatchability would, undoubtedly, be more marked should the birds used be fed on it from the time of hatching. Our endeavour was to determine the influence on the hatchability and chemical composition of the eggs from chicks fed from the time of hatching on diets which varied, as far as was possible, only in the source of protein supplement.

The Barred Rock chicks used for the experiment were grown in the Brooder house of the Poultry Department where each group had access to an outdoor concrete run which was completely screened in so that the chicks got an abundance of sunlight without contaminating the food supply. When the chicks were four months old ten pullets from each of the groups were removed to four specially constructed pens (28, 29, 30, 31) in the laboratory. The pens were constructed so that the birds were on wire, thus preventing access to their faeces, and so that trap-nests could be provided. The laboratory was heated, and the pens were situated so that they received no direct sunlight. Some minor modifications were made in the diet on which the birds had previously subsisted in an endeavour to make the only variable, as nearly as possible, the quality of

the protein from the different protein supplements. The birds in each group, however, continued to receive the same source of protein supplement as before. The fish meal, meat meal and tankage groups received in each case an amount of the protein concentrate which added 6 % crude protein ($N \times 6{\cdot}25$) to the diet. The low protein content of buttermilk powder made it impossible to incorporate an equivalent amount of crude protein to that added in the other diets and still keep the remainder of the diet the same in each case. Pen 28, therefore, received the equivalent of only 3 % of crude protein from buttermilk powder. The amount of mash containing 10 % crude protein was kept the same in each pen by varying the amount of dextrin. The amount of bone ash was varied to keep the total ash content of each diet approximately the same. The composition of the diets was as follows.

Table I. *Percentage composition of the diets.*

	Pen 28	Pen 29	Pen 30	Pen 31
Buttermilk powder	10·5	—	—	—
Fish meal	—	7·9	—	—
Tankage	—	—	10·7	—
Meat meal	—	—	—	10·5
Mash*	83·5	83·5	83·5	83·5
Cod-liver oil	2·0	2·0	2·0	2·0
Bone ash	3·5	3·1	2·5	2·5
Salt	0·5	0·5	0·5	0·5
Dextrin	—	3·0	0·8	1·0

* Ground yellow corn 70 parts; ground oats 30 parts; wheat middlings 50 parts; alfalfa meal 15 parts.

The birds commenced laying in January 1929 and the experiment was continued until August 30th, 1929. All the eggs laid, excepting those taken in the early summer for chemical studies were incubated and accurate incubation records kept. The results are given in Table IA.

Table IA. *Results of incubation trials.*

	Eggs set	Dead embryos	Infertile eggs	Chicks hatched	% of fertile eggs hatched
Pen 28. Buttermilk powder	496	213	163	120	36·0
29. Fish meal	633	318	75	240	38·4
30. Tankage	224	105	119	0	0
31. Meat meal	451	262	73	116	30·6
14. Cod-liver meal	676	91	167	418	82·0

The one male in each pen for the purpose of this part of the work was shifted to the next pen circularly each twenty-four hours so as to eliminate the fertility factor from this source as thoroughly as possible. The results of a hatchability study with cod-liver meal as the protein supplement are also included. These results (Pen 14) are not strictly comparable with those from the other pens, for while they represent the results from an equal number of pullets and at the same period of the year, the birds were not put on the experimental diet until four months before the hatching data were collected.

The cod-liver meal used had been freed by extraction from the large amount

of cod-liver oil which it contains. To do this a large copper extractor [Bryant, 1929] with a capacity of twenty pounds of meal was constructed on the Soxhlet principle, and the cod-liver meal extracted until fat-free. At first ether was used as the solvent, but later a distillate of solvent naphtha, all that distilled over under 90°, was used. The extracted cod-liver meal was then dried in the electric oven at a temperature sufficiently high to drive off all the solvent. The results represent the supplementary value of 3 % crude protein (N × 6·25) from oil-free cod-liver meal. These results are included at this time because of our particular interest in what influence this material might have on the hatchability of the eggs and because eggs from this diet were included in our analytical studies.

In Fig. 1 is shown the percentage distribution of the embryo mortality grouped into seven three-day periods.

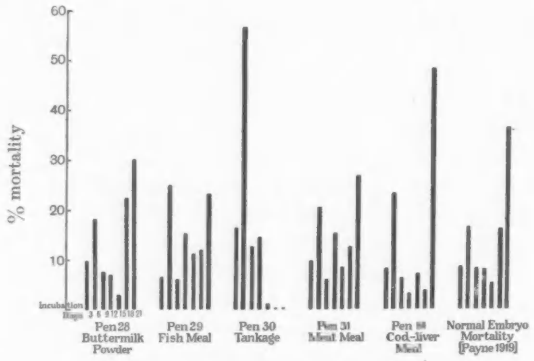

Fig. 1. The distribution of embryo mortality during incubation shown as 7 three-day periods.

The poor hatchability of the eggs from Pens 28–31 inclusive was somewhat surprising. It is of interest to compare these results with those of Graham and Smith [1929] where the same protein supplements, plus cod-liver oil, were used, but where mature pullets were placed on the experimental rations and where the conditions were such that the birds received a considerable amount of sunlight. The average results for February, March and April for both 1928 and 1929 in this experiment were as follows:

		% of the fertile eggs which hatched
Pen 4.	10 % buttermilk powder and cod-liver oil	69·5
28.	10 % fish meal and cod-liver oil	55·1
18.	10 % meat meal and cod-liver oil	67·4
30.	10 % tankage and cod-liver oil	38·7

While the results of these two experiments are not strictly comparable, they leave little doubt as to the effect which confinement of the birds and limitation

of their diet throughout growth and reproduction has on the hatchability of the fertile eggs.

The supplementary value of 3 % crude protein from buttermilk powder, so far as hatchability is concerned, was practically the same as that of 6 % crude protein from fish meal or meat meal. For some reason a very considerable number of the eggs from the buttermilk powder were infertile, which resulted in a lower hatch. Complete failure to hatch any of the eggs from the tankage group is noteworthy. The excellent hatch obtained from Pen 14, receiving fat-free cod-liver meal, would suggest that the inclusion of this meal in the diet of the laying hen has considerable value in improving the hatching quality of the eggs. Since the careful extraction of this meal with a fat solvent had removed the fat-soluble vitamins it would appear that the protein or inorganic constituents of cod-liver meal are of particular value in producing eggs of high hatchability.

The distribution of embryo mortality would also appear to vary with changes in the diet. That found with cod-liver meal and buttermilk powder is very similar to the "normal" distribution of embryo mortality during artificial incubation as described by Payne [1919]. In the meat meal and fish meal groups the embryo mortality is much more evenly distributed throughout the incubation period and, while showing the same sharply defined peaks around the 4th and 19th days of incubation as those described by Payne, there is also another critical period about the 12th day of incubation. In the case of the tankage group 56·5 % of the embryos died during the 4th, 5th and 6th days and only one embryo survived the 10th day of incubation.

Associated with this high mortality in the early period is the fact that the embryos were found in an extremely anaemic condition. On the 4th day of incubation many of the eggs from the tankage and meat meal groups, particularly the former, when candled, showed a shadow to one side of the germ spot. Such eggs were removed from the incubator, opened in warm saline and the embryo examined under the microscope. The embryo generally showed retarded development, and, while the heart beat was apparently normal, the circulatory fluid showed a lack of haemoglobin (pale) when compared with the normal, in many cases being almost colourless. Over 50 % of the embryos from the tankage diet were anaemic.

The composition of the proteins of eggs from the experimental diets.

That the composition of the egg may be influenced by the diet of the fowl is clearly evidenced from the investigation of Bethke, Kennard and Sassaman [1927], who have shown that the vitamin A and vitamin D content of egg-yolk is largely, if not entirely, determined by the amount of these substances present in the diet. It is also well known that the calcium carbonate content of the egg shell may be varied greatly by different nutritional regimes.

It is to be presumed, however, that the amino-acid make-up of the proteins of the white and the yolk would be the same, irrespective of the composition of the proteins in the diet and that a diet containing inadequate proteins would result only in a decreased egg production and not also in a change in the composition of the egg-proteins.

In this connection, Pollard and Carr [1924] report remarkable variations in the composition of the total protein of eggs from pigeons fed on diets restricted to a single seed grain for protein and vitamins. While the total nitrogen of the egg was fairly constant the distribution of the nitrogen in the egg-protein varied greatly in eggs from different diets [see McCollum and Simmonds, 1928]. Of particular significance is their observation that only the eggs which gave a high melanin yield were hatchable. Gortner and Holm [1920] have shown that the formation of melanin is dependent upon the presence of tryptophan in the protein molecule. It is possible, then, that the protein moiety in the diet may have a profound influence on the composition of the proteins of the egg, thus influencing protein metabolism during embryonic development and incidentally also influencing incubational mortality.

Prange, Hauge and Carrick [1927] have attributed the nutritional failure of meat meal to a deficiency of its protein in tryptophan. Their experiments consisted in first supplementing the meat meal ration with gelatin, and, as no better growth was procured, it was indicated that meat meal was deficient in either cystine or tryptophan, or both. Tyrosine was adequately supplied by the corn and wheat of the basal ration. In order to make a further elimination, the meat meal ration was supplemented by caseinogen, which had been previously subjected to alkali treatment completely to remove cystine. From the improved growth which resulted they conclude that the amino-acid deficient in meat meal may be tryptophan. Sendju [1925] found that after three days of incubation, at the moment of the sudden appearance of blood pigment, there is a great reduction in the tryptophan content of the egg. After still longer incubation, during which the bile pigment appears, there is a further lowering of the tryptophan content, which suggests that in the hen's egg both free and bound tryptophan are significant in the synthesis of blood and bile pigment. Minot and Murphy [1926] suggest that animal protein may favour blood formation and conclude that adequate proteins, as well as iron, are necessary for the formation of haemoglobin. They also suggest that tryptophan may have a special ability to enhance blood formation.

It is suggested that a deficiency of tryptophan in meat meal and tankage may result in a similar deficiency in the eggs from hens on diets containing these products as the chief source of the protein. Assuming that the embryo, as is believed in the case of the adult animal, has not the power to synthesise the pyrrole nucleus required for the building up of haemoglobin, and that tryptophan is the normal source of this organic nucleus during incubation, then it is not impossible that a tryptophan deficiency may result in the development of an anaemic embryo weakened in vitality which ultimately dies.

An examination of the egg reveals that the iron is nearly all located in the yolk, where it exists in the form of an organic complex which is not fat-soluble and has certain protein-like properties. This substance was first studied by Bunge [1884] and named "haematogen," and was later studied in more detail by Hugounenq and Morel [1905]. They regard haematogen as a prosthetic group in the vitellin molecule and as being the precursor of the haemoglobin formed by the chick during the incubation of the egg. This theory is considerably strengthened by the fact that coincident with the great increase in the haemoglobin content of the chick on the 14th day of incubation as shown by Sendju [1927] there is a corresponding sudden decrease in the vitellin-phosphorus of the yolk as shown by Plimmer and Scott [1909]. Decomposition of haematogen by acids according to Hugounenq and Morel splits off a black, iron-containing pigment (2·60 % Fe) which they named "haematovin" because it represents in the hen's egg, "an incompletely differentiated and embryonic state of haematin." It seems probable that the synthesis of haemoglobin has been partly completed in the body of the hen before the egg is laid, the embryo completing the formation of haemoglobin from this "preformed" compound. Anaemia in chick embryos may possibly be due to an inability to complete this synthesis or to a deficient supply of haematogen.

Copper according to Waddell *et al.* [1928; 1929, 1, 2] and Titus and Hughes [1928] must be considered another element necessary for haemoglobin synthesis, a deficiency of copper in the diet being a possible cause of nutritional anaemia. Drabkin and Waggoner [1929] on the other hand claim to have cured nutritional anaemia by means of a copper-free synthetic diet. It remains to be determined whether the copper content of the hen's egg is influenced by the copper content of the diet of the fowl, and whether nutritional anaemia in chick embryos from eggs deficient in copper may be possible.

Separation of egg-proteins.

The contents of the eggs obtained from the experimental birds in each of the pens described, *i.e.* Pens 28, 29, 30, 31 and 14, were separated into four protein fractions by the method of Plimmer and Rosedale [1925]. No attempt was made to separate the two proteins of the yolk, livetin and vitellin, the preparation being termed the egg-yolk-protein. The proteins of the white were separated into two fractions; one termed the egg-white-protein consisting of ovalbumin and ovoglobulin coagulated by boiling 0·4 % acetic acid, and the other the glycoprotein, ovomucoid, precipitated by absolute alcohol. To give sufficient material for analysis a composite of the egg-white-proteins from five eggs from each of seven birds in each pen was prepared. The same procedure was followed with the egg-yolk-proteins. In the case of the ovomucoid, where the yield per egg was small (0·1–0·3 g.), an amount insufficient for the purpose desired even with a 5-egg sample from an individual bird, composites of all the ovomucoid from each group were prepared. As we were particularly interested in the tankage group and as it was desired to obtain as many samples as

possible, although the number of eggs laid was limited, we prepared duplicate samples from the eggs of hen 1771 and triplicate samples from hen 1773.

As a control, the similar preparations of the proteins from five eggs, collected from five hens which were on range and which were receiving the poultry plant diet, were made and are designated the "normal."

The protein fractions separated were weighed, the average weight per egg in g. being as follows:

	Egg-white-protein	Egg-yolk-protein	Ovomucoid
Pen 28. Buttermilk powder	2·27	2·12	0·153
29. Fish meal	2·27	2·10	0·176
30. Tankage	2·11	2·14	0·155
31. Meat meal	2·38	2·19	0·195
14. Cod-liver meal	2·51	2·72	0·175

In the drying process on the glass plates, differences in the appearance of the yolks were observed. The yolks from any one hen, however, showed remarkable uniformity of appearance. Some would be transparent when nearly dry, others would show varying degrees of opacity. The depth of pigmentation was another variable property apparently uniform for an individual hen; and other such properties included size of yolk, and viscosity (whether "watery" or not so). It was noticed that the eggs of hen 535 on the cod-liver meal diet were large, and had very large yolks, which were light in colour and decidedly opaque, with a "watery" consistency. As a point of interest, the hatching record of this bird was consulted and it was found that in the month previous, namely April, 22 chicks had hatched from 23 eggs.

In the precipitation of the egg-white-protein with 0·4 % acetic acid it was found important to have the acid boiling briskly, to add the white slowly and with stirring, and to boil rapidly for at least three minutes. Even with these precautions poor separations were sometimes obtained, in which case repeated filtration and boiling were necessary. In the case of the hen 1787 this difficulty was unusually marked. Half an hour's boiling failed to bring about coagulation, nor was washing by decantation successful, a gelatinous mass being formed throughout the liquid. The separation was finally made by repeated washing and centrifuging, resulting in considerable loss. The hatching record of this hen revealed exclusively dead germs and infertile eggs.

A yellowish tinge in certain egg-whites was noticed to pass through with the ovomucoid fraction, being separated from this during the extraction with absolute alcohol.

Analyses of the protein samples. (Tables II, III and VII.)

The following determinations were made in duplicate.

(1) *Total nitrogen* (Kjeldahl).

(2) *Loss of weight on drying.* The samples as prepared contained a considerable amount of volatile matter in the form of alcohol and water. Weighed portions of about 0·5 g. were heated in the vacuum oven at 90°/5 mm. for six

hours, and the process repeated until two consecutive weighings differed by less than 0·5 mg.

Table II. *Analysis of egg-white-proteins.*

Sample	% loss in weight on heating in vacuum at 90°	Nitrogen (% dry weight)	Total amino-N in tryptic digest (% total N)
Normal	9·93	15·09	54·4
Pen 28. Buttermilk powder			
1751	8·06	15·13	55·0
1752	9·33	15·16	48·8
1753	9·01	14·90	51·2
1754	9·43	15·30	53·6
1755	10·86	15·51	52·4
1756	8·47	15·38	50·5
1758	9·07	15·05	50·9
Pen 29. Fish meal			
1762	9·18	14·89	53·1
1763	9·17	15·18	48·7
1764	10·73	15·60	48·4
1765	10·22	14·99	49·4
1766	9·28	15·38	48·1
1767	11·68	15·58	52·6
1768	10·95	15·10	51·1
Pen 30. Tankage			
1119	9·38	15·28	56·4
1771 (1)	10·25	15·55	53·9
1771 (2)	10·10	15·29	55·8
1773 (1)	12·68	14·91	53·2
1773 (2)	9·40	15·20	55·5
1773 (3)	8·64	15·50	55·1
1775	13·32	14·85	48·7
1776	9·50	14·76	53·5
1777	9·63	15·15	52·8
Pen 31. Meat meal			
1783	11·96	15·31	50·0
1784	9·95	15·15	52·1
1785	9·18	15·15	47·0
1786	10·42	15·14	47·0
1787	10·14	15·30	48·3
1789	9·96	15·15	49·6
1790	16·02	14·77	44·5
Pen 14. Cod-liver meal			
527	10·57	14·90	55·4
529	8·00	15·32	55·0
531	13·55	15·15	50·0
533	11·29	15·28	51·9
535	8·88	15·21	52·6
1012	10·70	15·19	48·6
1014	9·17	15·17	52·8

(3) *Total ash.* The ash content of the egg-white-proteins was so low (0·48 % in the case of the egg-white-protein from Pen 28) that it was not considered significant. The same applies to the ovomucoid samples. In the case of the egg-yolk-protein the ash content of the individual samples, calculated as % of the dry weight of protein, is included in Table III.

Table III. *Analysis of egg-yolk-proteins.*

Sample	% loss in weight on vacuum drying at 90°	Nitrogen (% dry weight)	Ash (% dry weight)	Total amino-N in tryptic digest (% total N)
Normal	8·22	14·39	4·73	43·1
Pen 28. Buttermilk powder				
1751	7·78	14·00	4·77	52·6
1752	7·90	14·38	5·16	50·1
1753	7·67	14·29	4·92	47·4
1754	7·37	14·30	5·19	47·2
1755	7·32	14·09	5·14	48·1
1756	7·91	14·48	4·87	37·6
1758	8·49	14·30	5·34	48·6
Pen 29. Fish meal				
1762	7·48	14·41	5·01	45·6
1763	8·09	13·95	4·52	39·4
1764	7·42	12·95	4·84	49·6
1765	8·18	14·11	4·67	47·7
1766	7·55	14·20	4·98	44·1
1767	8·15	14·35	5·06	43·5
1768	9·37	14·15	5·33	45·1
Pen 30. Tankage				
1119	7·58	14·60	4·90	47·2
1771 (1)	7·77	14·35	4·87	48·3
1771 (2)	7·65	14·20	4·84	50·7
1773 (1)	8·32	14·46	5·03	52·0
1773 (2)	8·78	14·65	5·02	54·1
1773 (3)	8·05	14·73	4·98	49·3
1775	8·72	14·66	4·83	50·8
1776	8·20	14·68	4·49	53·2
1777	8·23	14·69	5·06	54·1
Pen 31. Meat meal				
1783	7·70	14·35	5·03	50·5
1784	8·45	14·34	5·09	49·1
1785	8·28	14·32	5·06	45·2
1786	7·97	14·08	4·96	49·4
1787	7·75	14·21	4·94	46·1
1789	9·53	14·41	5·19	45·8
1790	8·24	14·39	4·97	45·5
Pen 14. Cod-liver meal				
527	7·65	14·46	4·79	51·0
529	6·72	14·55	4·70	51·8
531	6·78	14·61	4·94	57·5
533	8·64	14·50	5·18	52·4
535	8·25	14·69	4·69	48·7
1012	7·43	14·31	4·84	53·8
1014	8·19	14·55	4·71	48·0

(4) *Amino-acids.* The tyrosine, tryptophan and cystine content of these egg-proteins was determined. In view of the large number of determinations to be made it was considered that only enzymic hydrolysis of these proteins could be used and that the subsequent determinations of these amino-acids in the hydrolysates would have to be made by colorimetric methods. The following procedure was adopted.

Weighed quantities of the protein samples equivalent to 0·5 g. protein (N × 6·25) were transferred to 125 cc. Erlenmeyer flasks. A trypsin solution

containing 1·0 g. "Trypsin[1]" in 100 cc. of 0·4 % Na_2CO_3 was prepared. 25 cc. of this trypsin solution + 1 cc. toluene were incubated in tightly stoppered flasks for 10 days at 37·5°. During the first 24 hours the flasks were periodically gently shaken until the protein had gone into solution. At the completion of the digestion, the hydrolysates were acidified by adding 2 cc. 14*N* H_2SO_4 giving a total volume of 28 cc. in *N*/1 H_2SO_4. On adding 2 cc. 14*N* H_2SO_4 to the tryptic digests of the egg-yolk-proteins a heavy brown precipitate formed. This substance was believed to be haematovin (p. 1617). The acidified digests were centrifuged and the clear supernatant liquids decanted. That the tyrosine or tryptophan content of the digest had not been diminished by removing this precipitate was shown by the fact that the precipitate washed with *N* H_2SO_4 did not give the Millon or Hopkins-Cole reaction. The flasks were then placed in the refrigerator until required. Before commencing the amino-acid determinations the digests were shaken with a small amount of kaolin and filtered clear. Total amino-nitrogen was determined on 5 cc. of the hydrolysate using Van Slyke's macro-apparatus.

Tryptophan.

Tryptophan determinations were first made on 2 cc. of the tryptic digests of the egg-white-proteins and egg-yolk-proteins by the method of Ragins [1928]. Repeated determinations of the tryptophan content of the same sample of egg-white- or egg-yolk-protein by this method gave results which agreed very closely. It was found, however, that the tryptophan values of each seven egg-white- or egg-yolk-protein preparations from eggs from the same pen on the same diet were not constant, as we had anticipated, but fluctuated quite considerably. As a check tryptophan determinations were made on 2 cc. of the tryptic digests of the egg-white-proteins using the phenol reagent of Folin and Marenzi [1929, 1]. Direct determinations were also made on 1 g. samples of the egg-white-proteins by the method of May and Rose [1922] as modified by Boyd [1929].

Again variations were found in the tryptophan content of the seven egg-white-protein preparations of eggs from the same pen on the same diet, which were much greater than the experimental error of duplicate determinations on the same sample. Of particular significance, however, was the fact that there was no relationship between the fluctuations in the tryptophan content of the egg-white-proteins as determined by the three colorimetric methods, suggesting that the major factor contributing to these variations was the final colorimetric method of determination.

Table IV shows the results of these tryptophan determinations. Each value represents the mean tryptophan content of all the samples of egg-white- or egg-yolk-protein from each pen as enumerated in Table II. The probable error of the mean as determined by Peter's formula $\left(\text{P.E.} = 0{\cdot}8453.\frac{\Sigma V}{n\sqrt{(n-1)}}\right)$ is also given.

[1] Digestive Ferments Co., Detroit, Mich., U.S.A.

Table IV. *Estimation of tryptophan in egg-white- and egg-yolk-proteins.*

Results expressed as % of dry weight of protein.

Sample	Egg-white-protein			Egg-yolk-protein.
	By Ragins's reagent	By Folin's reagent	By May and Rose method	By Ragins's reagent
Pen 28. Buttermilk powder	1·37 ±0·019	1·27 ±0·018	1·53 ±0·048	1·49 ±0·019
Pen 29. Fish meal	1·31 ±0·028	1·20 ±0·011	1·53 ±0·020	1·47 ±0·016
Pen 30. Tankage	1·40 ±0·025	1·32 ±0·029	1·71 ±0·043	1·54 ±0·015
Pen 31. Meat meal	1·25 ±0·014	1·16 ±0·018	1·58 ±0·022	1·48 ±0·013
Pen 14. Cod-liver meal	1·51 ±0·015	1·20 ±0·040	1·76 ±0·014	1·53 ±0·023

Compared with the other methods the May and Rose determination was not so accurate. A few values had such a great variation from the mean value that it was considered best to discard these, using Wright's criterion of rejection, *i.e.* discarding all values showing variations from the mean greater than five times the probable error.

Because of the variability in the results of the tryptophan determinations on these egg-proteins from eggs from different hens on the same diet it was felt that, to detect slight variations in the amino-acid content of eggs from hens on different diets, more accurate methods of analysis would require to be evolved than the colorimetric methods which we purposed using. However, having found that changing the source of protein in the diet of the hen had a marked influence on the mortality of the embryo during incubation, our objective was to determine whether this effect was accompanied by gross changes in the composition of the egg-protein so far as the tyrosine, tryptophan or cystine content was concerned. It was concluded (see discussion) that the limits of accuracy of the colorimetric methods of analysis which we used would permit the drawing of definite conclusions in this regard.

The tryptophan content of the tryptic digests of the egg-yolk-protein and ovomucoids could only be determined by using Ragins's vanillin-HCl reagent. The colour developed with the phenol reagent was a different shade of blue from that developed by standard solutions of tryptophan or tyrosine. The colour developed by caseinogen, used as a standard, with *p*-dimethylaminobenzaldehyde was not comparable with the different shade of blue developed by the egg-yolk-protein or ovomucoid. The results of the tryptophan determinations on the ovomucoids is given in Table VII.

Tyrosine.

Tyrosine determinations were made on the tryptic digests by the method of Folin and Marenzi [1929, 1]. In determining the tyrosine content of the egg-white-proteins and the ovomucoids it was found that invariably in the unknown solutions after heating with $HgSO_4$ and cooling a white cloudy precipitate formed, which made the subsequent colorimeter reading impossible. To overcome this difficulty a slight modification of the procedure was made. After centrifuging the $HgSO_4$ precipitate containing the tryptophan, the tyrosine-containing supernatant liquid was transferred to a 50 cc. conical

centrifuge tube instead of a 100 cc. volumetric flask. After heating the unknown solution with $HgSO_4$ and cooling, a white cloudy precipitate formed. This precipitate was centrifuged and the supernatant liquid transferred to a 100 cc. volumetric flask. The precipitate was then washed with 10 cc. N H_2SO_4, centrifuged and the wash liquid added to the main solution. The remainder of the procedure was carried out in the usual manner, perfectly clear colorimetric readings being now obtained. The substance removed in the precipitate did not give any colour with Millon's reagent.

Variations in the tyrosine content of individual egg-protein samples from eggs from hens on the same diet were found as in the results of the tryptophan determinations. The mean tyrosine content of the egg-yolk- and egg-white-proteins from each diet and the probable error of the mean is given in Table V.

Table V. *Estimation of tyrosine in egg-white- and egg-yolk-proteins.*

Results expressed as % of dry weight of protein.

Sample	Egg-white-protein	Egg-yolk-protein
Pen 28. Buttermilk powder	4·96 ±0·037	4·85 ±0·054
Pen 29. Fish meal	5·02 ±0·029	5·06 ±0·029
Pen 30. Tankage	4·97 ±0·046	5·25 ±0·038
Pen 31. Meat meal	4·93 ±0·049	5·13 ±0·028
Pen 14. Cod-liver meal	5·14 ±0·055	5·26 ±0·014

The results of the tyrosine determinations on the ovomucoids are given in Table VII.

Cystine.

Recently Folin and Marenzi [1929, 3] have described a method for the preparation of the uric acid reagent completely free from the phenol reagent. The uric acid reagent was prepared according to the directions and was found to give no blue colour with the standard tryptophan solution. We could see no objection against determining the cystine content of these egg proteins by the use of this purified uric acid reagent with the tryptic digests. Glutathione, the only other substance which might have made the determination inaccurate, is not present in the unincubated egg. This procedure, of course, involves the assumption either that cystine is liberated quantitatively by tryptic digestion of these proteins or that comparatively the tryptic digestion (cystine liberated) of the different samples of the same egg-protein has proceeded to the same extent. Cystine determinations were made on a 5 cc. aliquot of the decolorised tryptic digests by the method of Folin and Marenzi [1929, 2]. In Table VI is

Table VI. *Estimation of cystine in egg-white- and egg-yolk-proteins.*

Results expressed as % of dry weight of protein.

Sample	Egg-white-protein	Egg-yolk-protein
Pen 28. Buttermilk powder	2·14 ±0·050	1·70 ±0·045
Pen 29. Fish meal	2·09 ±0·046	1·70 ±0·036
Pen 30. Tankage	2·22 ±0·046	1·66 ±0·041
Pen 31. Meat meal	1·98 ±0·028	1·72 ±0·020
Pen 14. Cod-liver meal	2·20 ±0·042	1·80 ±0·016

shown the average cystine content of the egg-white- and egg-yolk-proteins from each diet and the probable error of the average value.

The results of the cystine determinations on the ovomucoids are given in Table VII.

Table VII. *Analysis of ovomucoids.*

Sample	% loss in weight on drying *in vacuo* at 90°	Total nitrogen (% of dry weight)	Determinations on tryptic digest: Total amino-N (% of total N)	Tyrosine*	Tryptophan*	Cystine*	Total sulphur
Pen 28. Buttermilk powder	11·47	13·50	44·4	4·00	2·33	6·35	2·39
Pen 29. Fish meal	10·39	13·55	45·0	4·26	2·34	5·94	2·27
Pen 30. Tankage	10·36	13·48	45·7	3·99	2·09	6·36	2·24
Pen 31. Meat meal	10·08	13·51	45·9	4·22	2·36	5·82	2·24
Pen 14. Cod-liver meal	11·07	13·20	44·6	4·32	1·95	6·49	2·20

* Results expressed as % of the total protein (N × 6·25).

As a check on the fluctuations in the cystine content of these proteins, which is particularly marked in the case of the ovomucoids, total sulphur determinations were made on the ovomucoid by the sodium peroxide method [Assoc. of Official Agric. Chemists, 1924]. The disparity in the cystine content was not correlated with the total sulphur content as shown in Table VI. Cystine determinations have been made on the "normal" egg-white- and egg-yolk-protein and on a sample of purified caseinogen by H_2SO_4 hydrolysis and compared with the results obtained by tryptic digestion.

	Cystine: H_2SO_4 hydrolysate	Cystine: Trypsin hydrolysate
Caseinogen	0·30	0·38
Normal albumin	1·77	2·12
„ yolk	1·56	1·88

In each case the cystine content obtained by the same colorimetric procedure after H_2SO_4 hydrolysis was lower than that obtained by tryptic digestion. Plimmer and Lowndes [1927] have shown that cystine is changed by boiling with acids, losing 7 % of its nitrogen. The cystine content of ovomucoid is very high compared with that of the other egg-proteins. The method of expressing these results in Table VII makes the figure appear high. The corresponding results expressed as % of the original dry weight of material were 5·45, 5·04, 5·50, 5·00 and 5·60 respectively.

Determination of the iron content of the egg-yolk-proteins.

In determining the iron content of the egg-yolk-protein preparations we hoped to obtain an indication of any variation in the content of iron present in protein combination, a low iron content being a criterion of a low supply of this precursor of haemoglobin.

Iron determinations were made by a slight modification of the method of

Kennedy [1927]. As we desired figures for the ash content of our samples, the digestion method of Kennedy was not used. Instead, duplicate samples of about 0·5 g. of the egg-yolk-protein were ashed in platinum dishes at 500°–600° for at least 10 hours. The ash was weighed, then moistened with dilute HNO_3 and evaporated to dryness and again heated for a few minutes in the muffle, to ensure complete oxidation of all the iron. The cooled residue was then dissolved in 5 cc. of dilute HCl (1 : 1). The solution was then made up to 50 cc. and 10 cc. were taken for the iron estimation. The remainder of the determination was made by the thiocyanate method of Kennedy, 10 cc. of a solution containing 0·005 mg. Fe per cc. being used as the standard.

Table VIII. *Results of iron determinations on egg-yolk-protein.*

Sample	% iron in yolk-protein (dry weight) (1)	(2)	% iron in yolk-protein (N × 6·25)	% iron in yolk-protein ash
Normal	0·037	0·038	0·042	0·80
Pen 28. Buttermilk powder				
1751	0·057	0·064	0·068	1·28
1752	0·044	0·048	0·051	0·89
1753	0·057	0·055	0·063	1·14
1754	0·055	0·061	0·065	1·12
1755	0·057	0·060	0·066	1·15
1756	0·045	0·046	0·050	0·94
1758	0·049	0·046	0·053	0·90
Pen 29. Fish meal				
1762	0·050	0·055	0·059	1·06
1763	0·043	0·042	0·049	0·92
1764	0·041	0·041	0·051	0·85
1765	0·041	0·040	0·046	0·87
1766	0·034	0·033	0·038	0·67
1767	0·041	0·042	0·046	0·83
1768	0·044	0·044	0·050	0·84
Pen 30. Tankage				
1119	0·032	0·032	0·032	0·67
1771 (1)	0·039	0·032	0·035	0·78
1771 (2)	0·052	0·051	0·051	1·08
1773 (1)	0·052	0·049	0·050	1·01
1773 (2)	0·055	0·056	0·055	1·10
1773 (3)	0·056	0·057	0·056	1·14
1775	0·053	0·051	0·052	1·08
1776	0·050	0·049	0·050	1·11
1777	0·053	0·052	0·052	1·05
Pen 31. Meat meal				
1783	0·037	0·037	0·041	0·73
1784	0·043	0·044	0·049	0·86
1785	0·040	0·038	0·044	0·77
1786	0·040	0·040	0·045	0·82
1787	0·039	0·041	0·044	0·86
1789	0·029	0·031	0·033	0·57
1790	0·056	0·057	0·063	1·15
Pen 14. Cod-liver meal				
527	0·042	0·043	0·047	0·88
529	0·039	0·039	0·043	0·83
531	0·042	0·045	0·048	0·88
533	0·040	0·039	0·044	0·76
535	0·028	0·029	0·031	0·62
1012	0·048	0·049	0·054	1·01
1014	0·046	0·045	0·050	0·97

The results are shown in Table VIII. The iron contents of the diets fed was also determined and were as follows:

		% Fe in the diet
Pen 28.	Buttermilk powder	0·0053
Pen 29.	Fish meal	0·0068
Pen 30.	Tankage	0·0155
Pen 31.	Meat meal	0·0095

Determination of the copper content of eggs from different diets.

Owing to the relatively small proportion of copper in biological materials it was necessary to find a method which would bring the amount of copper contained in one egg well within the range of sensitivity of the method chosen.

The copper content of egg-yolk is given as 20 mg. per kg. of dry material by Fleurent and Levi [1920], as 2·5 mg. per kg. by McHargue [1925] and as 8·0 mg. per kg. by Lindow, Elvehjem and Peterson [1929] and as 1·8 mg. per kg. of fresh material (equivalent to 3·6 mg. per kg. of dry material) by Guerithault [1927]. The last mentioned author cites egg-white as being free from copper. Taking the last fact into consideration, and taking the weight of the yolk and white of an average egg as 45 g. of fresh material, 8 g. of which is yolk dry matter containing virtually all the copper, a yield of copper per egg of from 0·16 mg. to 0·02 mg. was anticipated.

For the estimation it was decided to try the method of Biazzo [1926] which has been used extensively by Elvehjem and Lindow [1929]. Unfortunately the high iron content of eggs was found to cause a discoloration of the chloroform layer making it difficult to compare with the standard. Elvehjem and Lindow give a somewhat unsatisfactory method for removing iron by the use of copper-free zinc. Instead of this the ash solution was treated with an excess of NH_4OH; the supernatant liquid then contains all the copper, free from iron. The procedure of Elvehjem and Lindow modified in this way was used and proved that a good recovery of copper from iron and phosphate mixture could be obtained.

Copper determinations were made on eggs from the birds in Pens 28, 29, 30 and 31. The eggs were weighed, boiled hard, peeled and the shell with its membranes discarded. The edible portion was ashed in platinum, dissolved in 1 : 1 HCl, excess of strong NH_4OH added, centrifuged to separate the gelatinous precipitate, the washings poured off and the precipitate redissolved in HCl and reprecipitated with NH_4OH, centrifuged and the supernatant liquid combined with the first ammoniacal solution. The process was repeated to ensure the recovery of all the copper from the gelatinous precipitate insoluble in the ammonium hydroxide. The combined ammoniacal washings were evaporated to a volume of about 5 cc. and washed into a 50 cc. stoppered cylinder. The determinations were completed by the method of Elvehjem and Lindow.

The results are given in Table IX. No attempt has been made to express averages. The reason for this is the great variation in copper content observed

in different eggs from the same hen on the same ration. For example, three eggs from hen 1789 gave a copper content of 0·04 mg., 0·09 mg., and 0·14 mg. respectively.

Table IX. *Results of copper determinations on eggs from different diets.*

Sample	Wt. of edible part of egg (g.)	% of ash in edible part	Total Cu (mg.)	Cu in edible part (mg./kg.)
Pen 28. Buttermilk powder				
1754	40·4	0·96	0·15	3·7
1754	45·1	0·92	0·05	1·1
1755	48·3	0·97	0·05	1·0
1758	40·5	1·02	0·10	2·5
1758	42·1	0·98	0·06	1·4
1758	44·5	1·00	0·05	1·1
1758	44·5	0·93	0·09	2·0
1759	52·4	0·86	0·11	2·1
Pen 29. Fish meal				
1762	43·5	0·98	0·07	1·6
1763	52·2	—	0·03	0·6
1763	52·0	0·99	0·05	1·0
1764	45·7	1·01	0·04	0·9
1765	46·7	0·88	0·11	2·4
1766	46·9	0·96	0·12	2·6
1766	50·3	0·91	0·06	1·2
1766	49·5	0·88	0·13	2·6
1768	46·2	0·88	0·05	1·1
1768	46·7	0·85	0·07	1·5
1768	46·4	0·86	0·09	2·0
Pen 30. Tankage				
1101	42·7	0·99	0·07	1·6
1101	42·6	0·98	0·05	1·2
1101	42·1	0·81	0·08	1·9
1101	37·5	1·08	0·08	2·1
1101	42·5	0·92	0·07	1·6
1771	42·0	0·97	0·14	3·3
1771	43·1	0·94	0·08	1·9
1771	42·1	0·91	0·08	1·9
1771	41·3	0·96	0·14	3·4
1776	42·3	0·94	0·08	1·9
1776	39·7	0·99	0·08	2·0
1776	40·3	0·96	0·17	4·2
1776	43·2	0·95	0·08	1·8
1777	44·3	0·95	0·15	3·4
1778	40·8	0·96	0·05	1·2
Pen 31. Meat meal				
1783	48·5	0·90	0·05	1·0
1783	46·8	1·05	0·08	1·7
1784	42·7	0·87	0·07	1·6
1784	52·4	0·86	0·09	1·7
1785	51·5	0·93	0·19	3·7
1785	43·8	0·92	0·06	1·4
1785	44·8	0·91	0·14	3·1
1786	39·7	1·13	0·04	1·0
1789	44·8	0·95	0·09	2·0
1789	38·8	0·97	0·14	3·6
1789	46·3	0·96	0·04	0·9

The copper content of the protein supplements and basal ration used in these experiments was also determined with the following results:

	Copper content (mg./kg.)
Buttermilk powder	3·8
Fish meal	4·2
Tankage	7·2
Meat meal	5·4
Cod-liver meal	13·35
Basal mash	3·4

Discussion.

There is no great difference in the weights of the different protein fractions from eggs in Pens 28, 29, 30 and 31. In the cod-liver meal group, Pen 14, the eggs gave a greater yield of egg-white- and egg-yolk-protein, particularly the latter. The egg-white- and egg-yolk-protein weights were very constant in the eggs from the different diets. There is no appreciable difference in the weights of the ovomucoid obtained from the different groups.

The total nitrogen content of the egg-white-, egg-yolk- and ovomucoid-protein in the different groups was fairly constant although discrepancies occurred which were much greater than could be accounted for. In the case of hen 1764 the nitrogen content of the egg-yolk-protein was much below the average, this figure having been confirmed by triplicate determinations. The total nitrogen content of the cod-liver meal ovomucoids was also slightly below the average, the nitrogen content of the other ovomucoid preparations being very constant. The ash content of the egg-yolk-proteins, Table III, as was the case with the total nitrogen, was fairly constant but varied to a greater extent than could be explained on the basis of experimental error, as duplicate determinations gave very close results. There was no correlation between the total nitrogen and the ash content, indicating that the slight variations were not due to differences in the purity of the preparations.

The tryptic digests of these proteins, the results of the analysis of which is given in Tables IV, V and VI, were all made with an aliquot of the same trypsin solution under identical conditions of temperature and time. The fluctuations in the total amino-nitrogen in the tryptic digests of the egg-white- and egg-yolk-protein (Tables II and III) are noteworthy. Duplicate determinations in no case varied more than 0·5 %. The total amino-nitrogen liberated by tryptic digestion of the ovomucoids was very constant.

The egg-white-protein of eggs of poor hatchability, *e.g.* from Pen 30, contained just as much tryptophan (Table IV) as the egg-white-protein of eggs of high hatchability, *e.g.* those of Pen 14. The variation with diet in the tryptophan content of the egg-white-protein as determined by the three methods is within the experimental error of the methods of analysis. No significant difference in the tryptophan content of the egg-yolk-protein from different diets (Table IV) was found by Ragins's method. Changing the tyrosine and tryptophan content of the diet of the hen by varying the source of protein did not result in changes of the same order in the content of these amino-acids in the egg-proteins. The variations in the tyrosine, tryptophan and cystine con-

tent of different samples of egg-white- and egg-yolk-protein from the same diet, as the result of sampling or inaccuracies in the methods of analysis, were just as great as the difference between different diets.

Of particular importance so far as this paper is concerned was the fact that there was no clear evidence that the diet of the hen had any influence on these values, and that the proteins from eggs of poor hatchability were found to contain just as much tyrosine, tryptophan and cystine as the proteins of eggs of high hatchability. It has to be admitted, however, that any slight changes in the amino-acid content of the egg-proteins could not be detected in these estimations and may possibly occur. The tyrosine, tryptophan and cystine content of the "normal" samples of egg-white- and egg-yolk-protein expressed as % of dry weight of protein was as follows:

	Egg-white-protein	Egg-yolk-protein
Tyrosine	5·22	4·82
Tryptophan by Ragins's reagent	1·41	1·48
" by Folin's reagent	1·30	—
" by May and Rose method	1·62	—
Cystine	2·12	1·88

There was no relationship between the variations in the tyrosine, tryptophan or cystine content of the ovomucoid (Table VII) from eggs from the different diets and the hatchability of these eggs. Taking into account the fact that only a single sample of ovomucoid from each diet was analysed, and the fluctuations previously found in the amino-acid content of the egg-white- or egg-yolk-protein from the same diet, the variations recorded in Table VII cannot be regarded as significant.

It will be noted that there is a difference in the tryptophan content of the egg-white-protein with different methods of determination. The method of May and Rose gave the highest tryptophan content while the results with the Folin and Marenzi procedure are uniformly lower than those obtained by the vanillin-HCl reagent of Ragins.

The great variation in the copper content, as shown in Table IX of eggs from the same hen on the same ration is noteworthy. The comparatively high copper content of buttermilk powder is of interest in view of the fact that the copper content of cow's milk is reported as 0·15 mg. per litre by Elvehjem, Steenbock and Hart [1929]. The contact with copper during the processing may raise the copper content of buttermilk powder within widely varying limits. The high copper content of cod-liver meal is of interest in view of the high copper content of mammalian livers. The copper content of tankage is quite considerable, probably because of the blood-meal which is incorporated in it.

Considerable variation in the iron content of the egg-yolk-proteins is shown in Table VIII. Variations from 0·031 % iron in the case of 535 to 0·066 % iron in the case of 1755, were obtained. Since the duplicates agree fairly closely these variations can be regarded as quite significant. There is also

a slight tendency for the iron content of the egg-yolk-protein from the eggs in the buttermilk powder group to be the highest of all. However, although the variations in iron content are considerable, there is no pronounced trend in the results which could be attributed to the influence of diet. The iron content of tankage is considerably greater than that of the other protein supplements, which is no doubt the result of the inclusion of blood-meal.

Compared with other feeds the protein supplements employed are liberally supplied with iron and copper, while the basal mash itself contains very considerable amounts of iron (0·0061 % Fe) and copper (3·4 mg. Cu per kg.). The possibility of nutritional anaemia in poultry as the result of a deficiency of iron or copper in the diet would seem to be extremely remote.

Summary.

(1) The source of protein in the diet of the hen was found to have a marked influence on the mortality of the embryos during incubation.

(2) No significant difference was found, however, in the composition of the proteins of eggs of poor hatchability so far as the total nitrogen, total amino-nitrogen, tyrosine, tryptophan and cystine content was concerned and the composition of the proteins of eggs of high hatchability. There was no clear evidence that the diet of the hen had any influence on these values.

(3) Total ash and iron determinations on the egg-yolk-proteins showed variations greater than could be accounted for by the limitations in the analytical methods used. There was little or no evidence, however, that the total ash and iron content of the egg-yolk-proteins was influenced by diet.

(4) The copper content of the hen's egg was found to be an extremely variable quantity.

(5) The results of these analyses of eggs from hens on different diets so far offer no explanation of the differences in their hatchability, nor do they throw any light on the cause of the anaemic state which has been found to be one of the major conditions associated with the mortality of embryos from tankage and meat meal eggs during the early stages of incubation.

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CLXXIX. VITAMIN B DEFICIENCY IN THE RAT. BRADYCARDIA AS A DISTINCTIVE FEATURE[1].

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Drummond and others have emphasised the non-specific character of the known signs of vitamin B deficiency. "Practically all the abnormalities that have been described as being due to vitamin B deficiency... can with one exception (head retraction or convulsions) be attributed solely to the loss of appetite and resulting inanition that follows withdrawal of the vitamin" [Drummond, 1926–7]. "In the case of rats at any rate the nutritive failure following a deficiency of the vitamin is virtually identical with that resulting from starvation" [Drummond and Marrian, 1926; see also Kon and Drummond, 1927, etc.]. In the present communication we are concerned with a distinctive abnormality which, although it occurs also in advanced starvation and inanition, cannot be adequately accounted for on the basis of the diminished food intake but appears to be due directly to the absence of the vitamin complex. The bradycardia which we have to describe is not seen in our control animals restricted to the same limited amount of food and it can be cured with dramatic rapidity by the simple administration of vitamin B when no increased food intake is permitted, or even during a period of complete abstention from food.

The rat has been so extensively employed in studying vitamin deficiency that reproducible results can now be readily obtained, but while results in the past have been judged mainly upon the appearance of the animal or upon body weight or such other gross changes as can be readily appreciated, attention now begins to be directed to changes in function. The heart presents the advantage that certain of its functions can be repeatedly and easily investigated. Electrocardiographic records can be obtained without undue disturbance of the animal, and from these the rate of the heart beat, the position at which it originates and the mode of spread of the contraction process through the heart can be accurately determined [Lewis, 1925]. The influence of abnormal diets upon two of its functions, namely rhythmicity and conductivity, can by this means be accurately studied. This consideration and the knowledge

[1] Communicated to the Biochemical Society, October 4, 1930 [Drury and Harris, 1930].

[2] Working on behalf of the Medical Research Council.

that cardiac abnormalities have been reported in clinical beriberi prompted us in 1928 to begin a comprehensive survey of the influence of various vitamin deficiencies or excesses upon these heart functions, and the present observations are an outcome of this work. Such studies may be expected to throw light not only upon the vitamin side of the question, but also upon the intimate functions of the heart itself.

Although the morphological cardiac changes which have been described in clinical cases of beriberi suggest the possibility of electrocardiographic irregularities also, none has, in fact, been recorded even during the height of the disease [Aalsmer and Wenckebach, 1929]. How far any such irregularities as may exist would be of significance here is difficult to decide, for other errors as well as simple vitamin B_1 deficiency are invariably present in clinical beriberi. The demonstration of heart block and bradycardia in pigeons fed upon polished rice of which a preliminary account has appeared [Carter and Drury, 1929] gave the study further impetus. The latter abnormality, however, is vagal in origin, whereas as we shall show the bradycardia in the rat is in no way dependent upon the integrity of the vagus nerves. To the present report we have appended a summary of our cardiographic findings in avitaminosis A, and D, and in hypervitaminosis D [for preliminary account see Harris and Moore, 1929, 1].

Method.

The electrocardiographic records have been obtained from unanaesthetised rats. In order to obtain satisfactory records the animals must be kept free from excessive movement, and to ensure this a specially designed board has been made upon which the animal can be quickly fixed and held secure upon its back[1]. The head of the rat is passed through an opening in a clamp, which is adjusted around the neck so that it is unable to withdraw it. The clamp consists essentially of two vertical brass plates with a triangular piece removed at the top of one and the bottom of the other. The upper piece, by means of a ratchet, can be raised or lowered, while the lower piece is fixed. By this means diamond-shaped openings of different sizes are obtained. This clamp is fixed on to a suitable board with side cleats. With the head firmly held in the clamp, threads with slip nooses are fixed to the fore and hind legs and fastened to the cleats. The rat, lying upon its back, is thus held firmly. After the observation is completed the threads are slipped off the limbs and the top vertical plate completely raised. The rat is allowed to free itself and is then picked up.

To obtain the electrical records two small needles are thrust under the skin of the right foreleg and the lower end of the thorax, about midway between the sternum and the left axillary line respectively[2]. These needles are passed about 2 mm. under the skin, and connected to a galvanometer string, the

[1] We are indebted to H. Hall of the Biochemical Laboratory for the construction of this apparatus.

[2] It was not considered necessary to record all the usual three leads, unless a definite and constant change was observed in this lead.

movement of which is recorded upon a moving paper camera. The point of insertion is marked by a dye so that the same position can be used at all subsequent examinations. Such needle electrodes are not "non-polarisable," and it is impossible accurately to standardise the string, and consequently the voltages of the curves cannot be compared, but information is available of the rate of the heart, the place of origin of the beat, the conduction between auricle and ventricle (the *P-R* interval) and conduction through the ventricular muscle (duration of the *QRS* complex) and the *T* wave. It has been found impracticable to use non-polarisable electrodes, owing to the high resistance of the fur and skin, which can only be lowered by prolonged soaking with saline. Comparative observations have been made, however, in anaesthetised rats of the curves obtained by the needle electrode method, with those obtained by non-polarisable electrodes fixed to right fore paw and left hind leg, and, as Table I (*a*) shows, both methods give identical results. In addition the *QRS* complex has been deformed by drugs and the records obtained show that the two methods in these circumstances also give similar measurements (Table I (*b*)). It can be safely assumed therefore that as far as the above measurements are concerned, the needle electrode gives the same results as the non-polarisable electrode, a conclusion which has been reached from the results already reported with the needle electrode in observations upon other animals [Verney, 1924].

Table I. *Comparison of needle electrode with non-polarisable electrode.*

(*a*) Normal rats anaesthetised.

Electrode used	Heart rate per minute	*P-R* interval in seconds	Duration of *QRS* interval in seconds
Rat 1. Non-polarisable	530	0·037	0·014
Needle	530	0·038	0·016
Rat 2. Non-polarisable	445	0·040	0·017
Needle	445	0·042	0·015

(*b*) Abnormal rats (anaesthetised) after injection of quinine sulphate.

Rat 3. Non-polarisable	381	0·052	0·032
Needle	381	0·051	0·031
Rat 4. Non-polarisable	290	0·060	0·038
Needle	290	0·058	0·037

In the experiments to be reported, rats of 40 to 80 g. initial weight on complete synthetic diet have been used; and although each experiment recorded has been controlled separately, a general consideration is desirable of the measurements from the electrocardiograms of young growing rats of such weights. The rats were held down upon their backs during the observation. The animal is of necessity subjected to the excitement of manipulation and is held in an unnatural position, but we have satisfied ourselves by a series of control tests that manipulation and the unusual position have very little influence upon the heart rate, and that the method employed gives an accurate estimate at least in so far as concerns young rats[1].

[1] Care is needed to avoid compression on the neck, which gives rise to a slow, irregular heartbeat, persisting for a long period.

The normal rat. In Table II the measurements of records from growing rats taken every seven days over a period of 42 days, and the average values of such measurements, are tabulated. It will be seen that the heart rate of each individual rat remains relatively constant over this period of time, the greatest variation in any one rat being 73 beats per minute. The average heart rate is from 503 to 530 per minute. The *P-R* interval is subject to very little variation, the greatest difference in any one rat being 0·0007 of a second. The average value is 0·036 to 0·041 of a second. The *QRS* interval also remains relatively constant. As it is impossible to ensure that the lie of the heart relative to the electrodes remains identical in the same rat on every occasion, slight alterations in the shape of the *QRS* complex occur and the greatest variations in the value are found when the form of the complexes is altered. The greatest difference in an individual rat is 0·005 of a second, while the average value is 0·009 to 0·012 of a second. The *T* wave shows little or no change throughout.

Table II. *Body weight in g., heart rate per minute, P-R and QRS intervals in seconds of growing rats on complete synthetic diet.*

Rat	Days ...	0	7	14	21	28	35	42
Female	Body weight	65	83	97	115	130	135	146
	Heart rate	490	480	500	514	540	529	490
	P-R	0·041	0·038	0·039	0·041	0·040	0·037	0·043
	QRS	0·014	0·014	—	—	0·013	0·012	0·013
Male	Body weight	56	75	97	110	137	153	166
	Heart rate	500	550	505	550	540	535	525
	P-R	0·039	0·035	0·033	0·036	0·036	0·034	0·035
	QRS	0·014	0·013	—	—	—	0·011	0·013
Male	Body weight	70	87	96	116	136	145	162
	Heart rate	525	491	533	525	540	562	533
	P-R	0·040	0·041	0·040	0·037	0·040	0·034	0·038
	QRS	0·013	0·010	0·012	0·008	0·010	0·012	0·011
Female	Body weight	78	87	95	106	117	124	142
	Heart rate	480	450	512	491	500	480	450
	P-R	0·040	0·042	0·037	0·039	0·040	0·037	0·042
	QRS	0·010	0·009	0·011	0·010	0·010	0·012	0·010
Female	Body weight	59	76	89	100	125	130	136
	Heart rate	550	533	540	558	534	533	500
	P-R	0·044	0·038	0·039	0·040	0·039	0·039	0·038
	QRS	0·010	0·011	0·008	0·009	0·010	0·009	0·007
Female	Body weight	57	67	77	84	100	103	105
	Heart rate	518	553	540	545	532	480	520
	P-R	0·041	0·034	0·037	0·038	0·039	0·035	0·033
	QRS	0·009	0·010	0·009	0·008	0·009	0·007	0·007
Average	Body weight	64	79	92	105	117	130	143
	Heart rate	513	510	525	530	530	520	503
	P-R	0·041	0·038	0·037	0·038	0·039	0·036	0·038
	QRS	0·012	0·011	0·010	0·009	0·010	0·011	0·010

The table shows that the values obtained for the heart rate, *P-R* interval, and *QRS* complex, remain relatively constant as the rats increase in weight from 64 g. to 143 g. Any definite change in any one of these values in rats of similar size occurring during a special experiment can, therefore, safely be ascribed to the experimental conditions imposed.

Observations have also been made upon rats dying from various causes other than vitamin deficiencies. Such moribund rats always exhibit a very slow heart rate which is often irregular and which sets in over a period of a few hours and reaches very low values; in fact, the lower the heart rate the nearer to death is the animal. The slow heart rate is sometimes associated with a lengthened *P-R* interval and on occasions with an incomplete heart block. This bradycardia of abrupt onset is also seen in dying rats on abnormal diets of all kinds and is an expression of the moribund condition of the animal and not a specific action of the diet.

Vitamin B deficiency.

The effect upon the electrocardiogram of absence of vitamin B from the diet has been tested upon a large number of rats. The composition of the diet was as follows: 20 % purified caseinogen, 55 % rice starch, 5 % salt mixture, 20 % arachis oil, supplemented with cod-liver oil, 2 drops per animal *per diem*. All the rats receiving this diet show a constant reaction. Coincidently with the fall in body weight, which usually begins after 2 weeks, a fall in heart rate sets in. During the next few days the heart rate rapidly decreases, reaching values of about 250–300 per minute. During this stage also the body weight steadily falls so that the two events run hand in hand (Fig. 1).

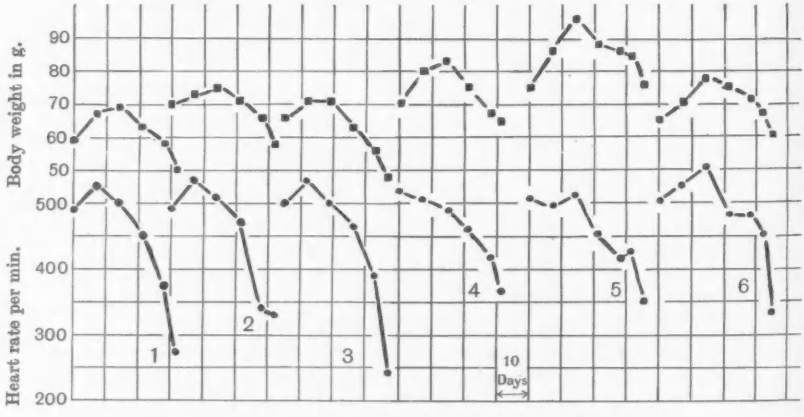

Fig. 1. Body weight and heart rate of rats fed upon synthetic diet free from vitamin B complex.

The measurements from six rats which are typical of all those receiving vitamin B-deficient diet are given in Table III.

It is seen from this table that although the heart rate falls considerably, even to half its original value, there is no change in the *P-R* or the *QRS* interval. This would indicate at first sight that the function of conductivity is unaffected, but it must be remembered that the slow heart rate allows a longer period of

Table III. *Body weight in g., heart rate per minute, P-R and QRS intervals in seconds of rats on a vitamin B deficient diet.*

Rat	Days ...	0	7	14	21	28	31	35
Female	Body weight	59	67	69	63	58	50	—
	Heart rate	490	525	500	450	375	273	—
	P-R	0·037	0·040	0·031	0·038	0·040	0·052	—
	QRS	0·013	0·013	—	0·010	0·015	0·019	—
Male	Body weight	70	73	75	71	66	58	—
	Heart rate	495	540	510	470	343	330	—
	P-R	0·042	0·034	0·034	0·042	0·034	0·039	—
	QRS	0·013	0·014	0·014	0·015	0·015	0·012	—
Female	Body weight	66	71	71	63	56	48	—
	Heart rate	470	540	500	465	390	240	—
	P-R	0·046	0·033	0·031	0·036	0·040	—	—
	QRS	0·012	0·015	0·013	0·014	0·012	—	—
Female	Body weight	70	80	83	75	67	65	—
	Heart rate	520	507	488	466	420	369	—
	P-R	0·039	0·040	0·042	0·043	0·038	0·037	—
	QRS	0·013	0·011	0·012	0·012	0·010	0·011	—
Male	Body weight	75	86	99	88	89	85	75
	Heart rate	507	496	514	450	415	428	343
	P-R	0·043	0·041	0·044	0·048	0·043	0·037	0·047
	QRS	0·009	0·011	0·011	0·009	0·009	0·011	0·010
Female	Body weight	65	70	78	75	71	67	60
	Heart rate	500	525	554	480	480	450	333
	P-R	0·037	0·037	0·036	0·038	0·040	0·036	0·046
	QRS	0·011	0·010	0·011	0·010	0·010	0·012	0·010

rest, so that an impairment of conduction might not be apparent under these circumstances. In some rats, a lengthened *P-R* interval is seen at the very low rates, but this is to be ascribed to the moribund condition of the animal rather than to any specific action of the deficiency, for it sets in suddenly, the lengthened intervals not being foreshadowed in the measurements obtained while the heart rate is steadily falling. The records show that there is no definite change in the size or direction of the *T* wave.

Controls with restricted food intakes. The close association of the fall in body weight with the onset of the bradycardia suggested the possibility that the cardiac change might be due not to any specific deficiency of the diet, but might occur in any condition in which the animal loses weight or might be due simply to insufficient food intake. To test this, a series of rats was taken, and grouped in pairs. One of each pair received the synthetic diet without vitamin B, the second of each pair (the control) received each day the same weight as the first ate the day previously, but of a complete synthetic diet containing 7·5 % marmite. Eight rats in all have been employed in these control tests, and all behaved consistently. The rats on the vitamin B-deficient diets soon showed some loss of appetite, ate less, and lost weight. The control rats on the restricted diets with marmite were found to lose weight likewise, in fact the weight curve of the paired rats usually coincided very closely. The electrical records showed that while the usual bradycardia developed in the rats on the vitamin B-deficient diet, the heart rate of the controls receiving limited amounts of the diets containing marmite remained within normal values (Table IV and

Fig. 2). It is evident from this experiment that the bradycardia is due to the absence from the diet of some factor present in marmite and is not due to inanition consequent upon the small intake of food. It is to be noted that the control rats upon the restricted diets may, if the intake of their pairs is very small, be brought ultimately to a moribund condition due to starvation. A bradycardia then sets in which confuses the result.

Table IV. *Body weights and heart rates of rats receiving (1) vitamin B-free diets and (2) identical amounts of complete synthetic diets.*

Rat	Days	0	7	14	21	28	Diet
Male	Body weight	79	85	85	79	62	Deficient in vitamin B
	Heart rate	514	540	525	430	300	
Male	Body weight	78	87	87	86	68	Restricted
	Heart rate	500	525	533	510	500	
Female	Body weight	71	73	73	67	55	Deficient
	Heart rate	488	500	471	408	300	
Female	Body weight	69	74	73	67	58	Restricted
	Heart rate	514	525	507	470	480	
Male	Body weight	73	58	65	58	44	Deficient
	Heart rate	525	500	480	450	320	
Male	Body weight	64	62	70	56	47	Restricted
	Heart rate	500	540	533	470	*500	
Male	Body weight	40	42	38	36	35	Deficient
	Heart rate	525	524	500	400	330	
Male	Body weight	40	41	37	37	38	Restricted
	Heart rate	540	545	510	510	510	
Male	Body weight	42	48	48	37	—	Deficient
	Heart rate	490	533	430	300	—	
Male	Body weight	46	48	48	45	41	Restricted
	Heart rate	525	500	553	550	536	
Female	Body weight	42	45	45	40	36	Deficient
	Heart rate	525	524	470	380	330	
Male	Body weight	43	49	51	47	41	Restricted
	Heart rate	540	545	560	540	504	

* 25th day.

Cure of the bradycardia with various sources of vitamin B[1]. When an extract of marmite[2] in doses of 0·75 cc. *per diem* was added to the diet of vitamin B-depleted rats with typical bradycardia, the heart rate rose rapidly and on the next day normal values were always recorded. In such experiments the animal quickly regained its appetite, its general condition improved, and it gained weight rapidly. In order to confirm the conclusion given in the last paragraph it was necessary to discover whether the rise in heart rate was due to any increase in the amount of food taken, or to the specific action of the vitamin, or possibly to inorganic substances present in the marmite. The influence of the amount of food taken can be eliminated by restricting the animals to the same limited amount of food as they had been consuming during the few preceding

[1] We are indebted to Miss Janet Clay for her assistance in this part of the work.

[2] [Harris and Moore, 1928, p. 1464.]

days when on the vitamin B-free diet, and that of the inorganic factors by using a very active yeast concentrate[1]. In an experiment conducted on these lines we find that the animal's condition and appetite improve, but the restriction in the food allowance results in the weight remaining stationary or rising only slightly (Fig. 2, Nos. 1 and 2). The heart rate, on the other hand,

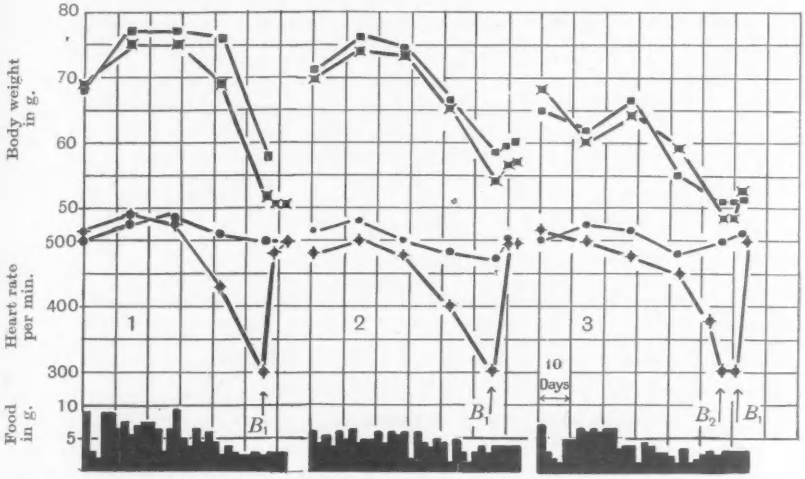

Fig. 2. Body weight and heart rates of paired rats, one upon a vitamin B-deficient diet, the other restricted to the same weight of a full synthetic diet. At B_1 in each instance, the vitamin B-depleted rat was given 0·15 cc. daily of yeast concentrate, no increase in food intake being permitted. At B_2 3 cc. of autoclaved marmite extract were given daily = 1 g. marmite. The food intake is shown at the bottom of the figure.

■ ●—Restricted diet. ✦ ✱—Vitamin B deficient diet.

returns to its normal value (Fig. 2). It would appear therefore that the amount of food taken is not the essential factor in the return of the heart rate to normal. Moreover, a concentrate of yeast is capable of restoring the normal heart rate without the occurrence of any coincident change in body weight.

Further evidence that an increased food intake is not essential for the alleviation of the bradycardia is provided by the demonstration of a return to normal rate within 3 to 4 hours following the administration of the concentrate, all food being withheld during the period. In studying the cardiac events immediately following the administration of substances rich in vitamin

[1] For this concentrate and the information concerning it we are indebted to Mr Guha of the Biochemical Laboratory, Cambridge. The concentrate was prepared as follows. Brewer's top yeast was extracted first with 97 % alcohol and then with 50 % alcohol. The two extracts were mixed and, after removal of alcohol, precipitated with neutral lead acetate. The filtrate, after removal of lead by hydrogen sulphide, produced good growth in rats, kept on a vitamin B_1-deficient diet, in a daily dose of 0·15 cc. which contained 35 mg. organic and 4·5 mg. inorganic matter. That the curative action is not due to the presence of salts is evident from the minuteness of the amount present in the active concentrate which is considerably exceeded by the amount consumed in the basal diet.

B_1, marmite and wheat germ as well as the yeast concentrate have been used. In these experiments the heart rate has been recorded before the administration of the substances and at hourly intervals afterwards. To eliminate any factor due to the intake of food after giving the concentrate, some of the rats have been allowed water only and no food during the first 6 hours following the administration of the concentrate. The concentrate has been used in two ways. Either 0·15 cc. has been pipetted into the mouth or the same amount has been diluted to 0·5 cc. with saline and injected into the peritoneal cavity. The wheat germ was readily and quickly eaten. The marmite was pipetted directly into the mouth. The results of the experiments in which only water has been allowed after administration of the vitamin are shown in Table V and Fig. 3, Nos. 1 and 2.

Table V. *Cure of bradycardia by administration of vitamin B during a period of starvation.*

	Heart rate per minute		
	Before	After*	Substance and dose
Male	300	300 ($\frac{1}{2}$) 400 (1) 430 ($2\frac{1}{2}$) 500 (23)	0·15 cc. concentrate by mouth
Male	380	400 ($\frac{1}{2}$) 408 ($1\frac{1}{2}$) 410 ($2\frac{1}{2}$) 500 (23)	,, ,,
Female	350	415 (1) 440 (2) 450 (3) 505 (4) 510 (24)	Same dose injected intraperitoneally
Male	350	435 (1) 470 (2) 500 (3) 510 (4)	,, ,,

* Time in hours after administration of substance in brackets.

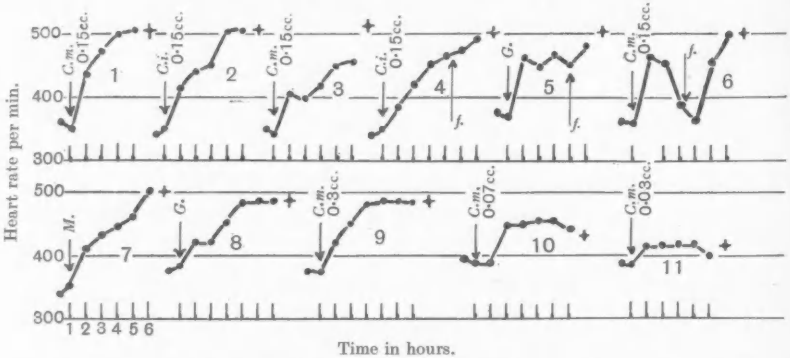

Fig. 3. Heart rates of vitamin B-depleted rats, before and for several successive hours after administration of one dose of a substance rich in vitamin B_1.
C.m. = yeast concentrate by mouth. *C.i.* = concentrate injected peritoneally. *G* = wheat germ. *M* = extract of marmite. Nos. 1 and 2: no food available till six hours after administration. Nos. 4, 5, 6: no food offered till arrow marked *f*. Nos. 3, 7, 8, 9, 10, 11: food available throughout. Water available in every instance.

From these measurements it is seen that the heart rate has risen considerably within the first hour; a few experiments indicate that at the end of the first half-hour there is an appreciable rise in rate, and that within 3–6 hours normal values are being recorded. The withholding of food after the administration of the concentrate may however sometimes give rise to certain

undesirable features. At the late stage of the avitaminosis at which it is selected, the rat is often feeding badly, but is feeding frequently; so that if food is withdrawn it quickly passes into a moribund condition due to starvation. This in itself gives rise to a bradycardia which confuses the result. This is clearly indicated by the experiments recorded below (Table VI, Fig. 3, Nos. 4, 5 and 6).

Table VI.

	Heart rate per minute: Before	After*	Substance and dose
Male	275	350 (1) 350 (2) 280 (3) Animal moribund	0·75 cc. marmite extract
Male	375	470 (1) 465 (2) 400 (3) Food given 370 (4) 460 (5) 500 (6) 500 (24)	0·15 cc. concentrate by mouth
Female	360	465 (1) 450 (2) 470 (3) 450 (4) Food given 480 (5) 500 (6) 525 (24)	0·75 cc. marmite extract

* Time after administration of substance in hours in brackets.

The recovery therefore is best studied in animals which are allowed restricted food, usually 4–6 g. daily, and which are able to eat at any time, if they desire. The measurements from such experiments (Table VII) show that full recovery is usually reached in 3–5 hours but that in some rats it may be delayed. (If a small dose of the concentrate is given, the heart rate fails to reach normal values, and reaches values which are approximately related to the size of the dose; see below.)

Table VII. *Curative experiments with restricted food allowance.*

	Heart rate per minute: Before	After*						Substance and dose
Male	360	405 (1)	400 (2)	420 (3)	450 (4)	455 (5)	525 (24)	—	0·15 cc. concentrate by mouth
Female	350	410 (1)	430 (2)	445 (3)	460 (4)	500 (5)	500 (24)	—	0·75 cc. extract marmite
Male	380	420 (1)	420 (2)	450 (3)	480 (4)	480 (5)	480 (6)	480 (24)	½ g. wheat germ
Male	375	420 (1)	450 (2)	480 (3)	490 (4)	480 (5)	480 (6)	480 (24)	0·30 cc. concentrate by mouth
Female	385	385 (1)	450 (2)	450 (3)	455 (4)	455 (5)	455 (6)	435 (24)	0·07 do.
Male	385	415 (1)	420 (2)	420 (3)	420 (4)	400 (5)	420 (24)	—	0·03 do.

* Time after administration of substance in hours in brackets.

The heart rate of rats cured in the manner described after remaining within normal limits for, say 1–3 days, begins to fall again, and 3 to 4 days later it is once more between 300 and 400 per minute. This enables the same dose to be repeated, or another substance to be tried. This has been performed upon several rats and the results are charted in Fig. 4.

Autoclaved marmite has been tried in a few cases as a substitute for vitamin B_1 concentrates (Fig. 2, No. 3 and Fig. 4, No. 6), but in no case has the bradycardia been cured. In one instance we used a preparation kindly provided by Mr Guha: the marmite had been autoclaved for 1½ hours at 115° at p_H 9·0; the amount given (3 cc.) was known to be adequate as a source of

vitamin B_2, from separate control experiments[1]. In another experiment the autoclaved marmite had been given us by Prof. R. A. Peters; this sample had been autoclaved for 1 hour at 115° at p_H 9·0.

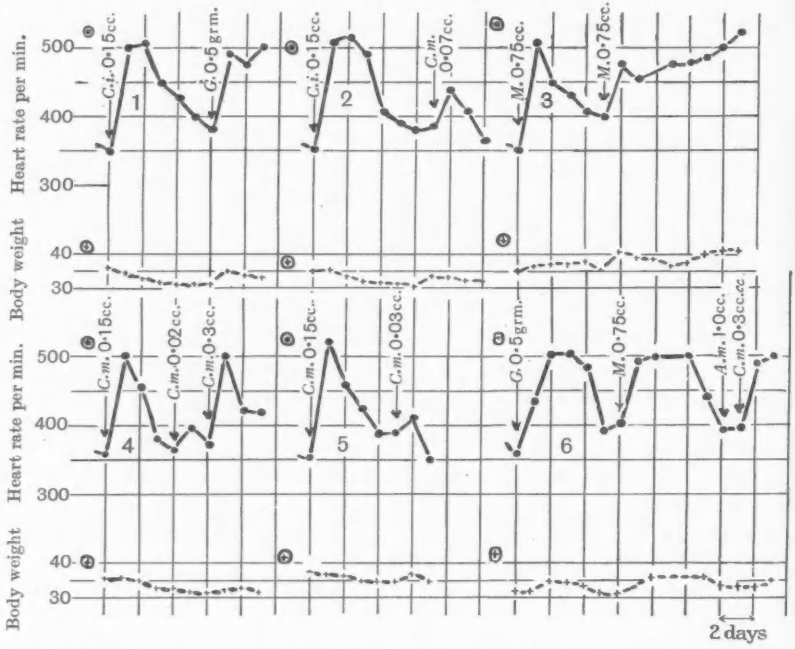

Fig. 4. Daily heart rates before and after the administration of single doses of substances rich in vitamin B_1.

C.m. = concentrate given by mouth. *C.i.* = concentrate injected intraperitoneally. *G* = wheat germ. *M* = extract of marmite. *A.m.* = autoclaved marmite. The heart rates and body weight before feeding the vitamin B-deficient diet are encircled.

With regard to the curative experiments with vitamin concentrates it may be undesirable to draw too definite conclusions of a qualitative nature without the use of a still larger number of animals, but there are certain points regarding the relation between the dosage and the degree of response which seem of interest. (1) After intraperitoneal injections of 0·15 cc. of the concentrate (Fig. 4, Nos. 1 and 2) the heart rate is within normal values for 2 days; after giving the same doses by mouth (Fig. 4, Nos. 4 and 5) normal values are only found during the day after. (2) To rats Nos. 4, 2 and 5 (Fig. 4) the concentrate was given on the same day in doses of 0·3, 0·07, and 0·03 cc. respectively; on the next day the heart rates were respectively 500, 430 and 410. The recovery of these rats during the hours immediately following the administration is shown in Fig. 3, Nos. 9, 10 and 11. It is evident that the degree of recovery is

[1] This sample contained very little vitamin B_1.

related to the amount of vitamin which is available, a result which was anticipated, since the fall in heart rate is gradual in onset.

The nature of the bradycardia in vitamin B deficiency.

The electrocardiographic records show that when the heart is beating at rates around 350, the bradycardia is sinus in origin. When lower rates are reached the *P* wave is lost, and it becomes difficult to decide whether A.V. rhythm has set in or not, no records having been obtained showing the change from the sinus bradycardia to the A.V. nodal bradycardia. In consequence of this, observations have been confined to animals in which the bradycardia is definitely sinus in origin.

It has already been reported [Carter and Drury, 1929] that pigeons fed on polished rice develop a heart block which is associated with a sinus bradycardia, and that this can be abolished by vagal section or atropinisation. It was anticipated that the sinus bradycardia in the rat would likewise be of vagal origin, but vagal section or complete atropinisation fails to return the heart rate to normal values.

The vagi have been sectioned in three animals and atropine injected into several others. Under urethane and ether anaesthesia the vagi were dissected in the neck. Records of the heart rate were then taken and the vagi sectioned, one after the other, records being taken at varying intervals afterwards (Table VIII).

Table VIII. *Bradycardia of avitaminosis B after vagal section.*

	Heart rate per minute				
	Before	Both vagi sectioned*			
1	300	330 (1)	350 (3)	330 (5)	328 (7)
2	350	320 (1)	310 (2)	300 (3)	290 (5)
3	320	310 (1)	310 (2)	300 (3)	290 (5)

* Time in minutes after section in brackets.

It is apparent that vagal section although producing in some rats a slight increase in rate by removal of vagal tone fails to restore the heart rate to the normal values. These observations are supported by the subcutaneous injection of atropine sulphate (Table IX).

Table IX. *Bradycardia of avitaminosis B after injection of atropine.*

	Heart rate per minute					
	Before	After*				Dose
1	343	330 (1)	323 (3)	333 (5)	—	0·2 cc. of 0·1 % solution†
2	250	240 (3)	240 (6)	230 (9)	225 (15)	0·2 cc. of 0·1 % solution‡
3	378	360 (1)	360 (3)	360 (5)	360 (8)	„ „

* Time in minutes in brackets. † Unanaesthetised. ‡ Anaesthetised.

Experiments were also performed which showed that after subcutaneous injection of 0·2 cc. of a 0·1 % solution of atropine neither electrical stimulation

of the vagus nerve, nor the intravenous injection of acetylcholine has any influence upon the heart rate; the vagus nerves being completely paralysed.

It is evident therefore that the sinus bradycardia, which occurs when rats are fed on a diet free from vitamin B, is in no way dependent upon the integrity of the vagus nerves, and in this respect differs from the heart block seen in pigeons fed upon polished rice.

Evidence has also been obtained by the fact that the bradycardia is not influenced by the injection of barium, that it is not related to the bradycardia produced by the injection of muscle adenylic acid [Drury and Szent-Györgyi, 1929].

In studying these bradycardias it is important that the temperature of the animal should be borne in mind and be kept carefully controlled during the observations. The rectal temperatures of rats depleted of vitamin B is often 34°–35° and, if the animal is moribund, may be even lower, and is readily influenced by external temperature. Experiments have been performed upon anaesthetised, normal, and vitamin B-deficient rats to determine the influence of rectal temperature upon the heart rate, and it is found that between the temperatures of 30°–40° a rise of one degree causes an increase in heart rate of approximately 15 beats. While the lowered temperature of the animal may conceivably help in producing the very low heart rates such as 250 per minute, which are occasionally seen, it is not responsible for the bradycardia with heart rates of 300 per minute and over, for these rates have been consistently found in rats whose rectal temperature was not allowed to fall below 37°–38°.

Vitamin A deficiency.

Baude and Deglaude [1924] have reported that there is no change in the heart rate in rats fed upon diets deficient in vitamin A, and our results serve to confirm and amplify their findings.

We have carried out tests upon two series of rats. The basal diet had the same composition as previously given, with the addition of 7·5 % of marmite extract as a source of vitamin B and 1 drop per animal per day of "radiostol" for vitamin D. All the experimental animals developed typical avitaminosis A.

Both series showed that the absence of vitamin A had no influence upon the rhythm of, or the conduction in, the heart, at least until the animal was almost *in extremis*. No change in the *T* wave was seen. The measurements from two rats which are typical of the series are given in Table X.

The controls of these series (receiving vitamin A in the form of a small supplement of red palm oil or carotene, or "radiostoleum" in place of "radiostol") increased in weight from an average of 52 g. to 148 g., and showed no change in the heart rate, the *P-R* or the *QRS* interval.

Table X. *Heart rates, etc., of rats receiving vitamin A-deficient diet.*

Table X. *Heart rates, etc., of rats receiving vitamin A-deficient diet.*

	Days ...	0	7	14	21	28	35	42	49	56	63	70	77	80
Female	Body weight	55	69	83	96	105	115	122	131	136	129	124	120	Dead
	Heart rate	520	600	600	565	525	571	553	543	533	525	500	480	
	P-R	0·037	0·033	0·033	0·037	0·037	0·033	0·034	0·034	0·032	0·035	0·036	0·037	
	QRS	0·013	0·009	0·010	0·009	0·009	0·008	0·007	0·008	0·008	0·015	0·013	0·008	
Male	Body weight	63	85	98	114	131	145	156	168	174	175	175	Dead	
	Heart rate	507	600	545	550	533	545	543	533	521	514	514		
	P-R	0·039	0·035	0·035	0·036	0·036	0·036	0·036	0·038	0·035	0·037	0·038		
	QRS	0·013	0·010	0·011	0·010	0·009	0·009	0·008	0·009	0·008	0·009	0·012		

Table XI. *Heart rates, etc., of rats on a rachitogenic vitamin D-deficient diet.*

	Days ...	0	7	14	21	28	35	42	49	56	63	70	77	84
Male	Body weight	73	80	88	99	105	110	114	124	132	129	132	130	130
	Heart rate	480	557	533	514	500	500	491	514	507	487	480	480	500
	P-R	0·038	0·033	0·036	0·035	0·039	0·036	0·036	0·037	0·037	0·038	0·038	0·036	0·035
	QRS	0·014	0·010	0·011	0·009	0·009	0·010	0·009	0·009	0·009	0·010	0·013	0·017	0·013
Female	Body weight	50	53	60	62	65	72	77	84	87	89	94	97	98
	Heart rate	545	540	525	540	525	566	533	545	550	520	562	514	525
	P-R	0·035	0·035	0·036	0·033	0·037	0·033	0·035	0·036	0·035	0·034	0·034	0·040	0·035
	QRS	0·013	0·009	0·011	0·011	0·008	0·009	0·009	0·008	0·008	0·013	0·011	0·015	0·013

Table XII. *Heart rates, etc., in hypervitaminosis D.*

	Days ...	7	14	21	28	35	42	49	56	63
Male	Body weight	72	74	74	69	69	73	73	63	64
	Heart rate	533	533	540	533	480	500	480	450	415
	P-R	0·036	0·037	0·034	0·034	0·037	0·040	0·034	0·036	0·044
	QRS	0·015	0·013	0·009	0·010	0·008	0·009	0·010	0·009	0·010
Female	Body weight	86	84	84	87	85	91	92	83	76
	Heart rate	562	533	500	525	480	500	480	430	471
	P-R	0·037	0·036	0·036	0·034	0·036	0·039	0·034	0·041	0·039
	QRS	0·013	0·011	0·009	0·009	0·009	0·008	00·07	0·008	0·010

Table XIII. *Electrocardiographic records of rats receiving diet deficient in vitamins A and D.*

	Days ...	0	7	14	21	28	35	42	49	56	63
Male	Body weight	60	63	81	99	113	123	133	137	129	—
	Heart rate	500	600	566	570	545	560	572	507	514*	—
	P-R	0·040	0·035	0·037	0·036	0·038	0·031	0·030	0·036	—	—
	QRS	0·013	0·010	0·008	0·008	0·009	0·007	0·007	0·008	—	—
Female	Body weight	55	60	73	85	95	95	100	99	92	90
	Heart rate	480	600	600	565	525	525	549	505	500	450†
	P-R	0·039	0·036	0·029	0·035	0·037	0·039	0·032	0·035	0·034	0·039
	QRS	0·009	0·009	0·008	0·008	0·008	0·010	0·007	0·008	0·008	0·015

* Died 58th day. † Died 64th day.

Vitamin D.

Rats were placed on a Steenbock rachitogenic diet devoid of vitamin D. The measurements of two rats, typical of the series, are given in Table XI. Both these rats were killed on the 84th day and *post mortem* and histological examinations showed them to be suffering from typical severe rickets. The electrocardiographic measurements throughout show no abnormal variation, even at the stage when severe rickets had developed, and it appears evident that absence of vitamin D has no influence upon the rhythm of, or conduction in, the heart. No change in the *T* wave was found.

Hypervitaminosis D.

Agduhr and Stenstrom [1929] have reported changes in the ventricular portion of the electrocardiogram of mice fed upon excess of cod-liver oil which they consider typical of the condition and which they state can be related to the morphological changes in the heart muscle. We have ourselves been interested mainly in hypervitaminosis D induced by excessive administration of irradiated ergosterol, rather than in the effects of large doses of cod-liver oil which appear to have a different action [Harris and Moore, 1928; 1929, 1, 2]. The amount of irradiated ergosterol given was generally about 5 mg. (about 5×10^4 Coward units of vitamin D). The measurements of two rats typical for the series are given in Table XII.

It is seen that the heart rate tends to fall somewhat towards the end of the experiment. The fall in rate is in many cases quite slight, but all the rats on such diets at the end have heart rates which are below or on the lower limits of normality. The form of the electrocardiogram remains unchanged throughout. *Post mortem* examination of the rats showed the usual extensive deposits of calcium in the upper part of the aorta, and in the kidneys, etc. [Harris and Moore, 1929, 1].

Simultaneous deficiency of vitamins A and D.

A series of rats has been placed upon a diet which was deficient in both vitamins A and D. Measurements of two rats typical of the series are given (Table XIII). No change is found in the measurements or in the *T* wave.

Discussion.

Of the vitamin deficiencies tested, A and D, separately and combined, appear to exert no characteristic influence upon the rhythm of or the conduction in the heart or upon the *T* wave. Excess of vitamin D produces a very slight but definite bradycardia.

Deficiency of vitamin B has, however, a striking influence upon the rhythm, the heart rate being reduced from the normal values of 500–550 per minute to 350–300 per minute. The first point which emerges from our experiments is that the bradycardia although coincident in degree with the fall in body weight is

not part and parcel of such loss of weight, or of the diminished food intake, for control rats upon restricted diets losing weight at approximately the same rate fail to develop bradycardia until they are moribund. Again the bradycardia can be cleared up with rapidity by the administration of extracts rich in vitamin B, even during a short period of complete deprivation of all food, water alone being allowed. Indeed, a prolonged recovery of normal heart rates can be effected without gain in body weight by administering the vitamin while restricting the food intake to the amount consumed during the later stages of the avitaminosis. Reviewing our evidence we feel, therefore, that the bradycardia is not entirely a secondary effect resulting from diminished appetite and consequent inanition. A bradycardia, possibly indistinguishable experimentally, certainly develops when an animal is moribund as a result of extreme inanition or from other reasons. In the later stages of avitaminosis the animal is, it is true, not far removed from a moribund state due to starvation, but, as we have shown, the bradycardia begins to develop at a time long before the failing appetite can be regarded as a sufficient cause, and it can be terminated in the absence of food. In short we have a sign of advanced inanition, for which the degree of starvation is in itself an inadequate explanation. The growth failure, on the other hand, would seem to be adequately accounted for simply by the diminution in the weight of food eaten. The possibility may be borne in mind of the loss in appetite being secondary to the bradycardia.

It seems probable that the bradycardia (curative) test will prove of value in future work on the separation of the constituents of vitamin B. It is as sensitive as, and more specific than, a rat-growth test, and can be concluded in fewer hours than the latter requires days. We have seen that various sources of the vitamin B complex contain the antibradycardia substance, so that when only a rough assay for the vitamin complex as a whole is desired, without special reference to a particular constituent, the convenience of the new technique is again apparent.

As to the relation of the vitamin to the mechanism underlying the bradycardia, it is impossible, at this stage, to draw any definite conclusions. Normal cardiac rates are not reached for at least 3 hours after administration. The fact that several hours elapse suggests the possibility that certain changes must take place in the body before the rhythmicity is normal again. If the vitamin supplied a link in the metabolic process underlying rhythmicity one might expect the recovery to be even more abrupt, but, on the other hand, the recovery is too rapid for any organic degenerative process to underlie the impairment. A possible correlation may exist between the bradycardia and the accumulation of lactic acid in vitamin B depletion [Bickel, 1924, 1925; Collazo, 1923]: this accumulation is, according to Peters [1929], responsible for the opisthotonos, and its removal is accelerated by vitamin B. The well-known hypertrophy of the suprarenal gland seen in vitamin B deficiency [Marrian, 1928] might also be legitimately thought of here, with the possibility of some irregularity in the secretion of adrenaline.

We have seen that the component of the vitamin B complex responsible for the cure of the bradycardia is present in certain vitamin B_1 concentrates and that like vitamin B_1 it is comparatively heat-labile. Again certain sources of vitamin B_2 are inactive. But we consider that we have not yet examined a sufficient variety of preparations to enable us to identify it with a particular constituent of the complex, and in fact owing to the present position of the vitamin B problem we are of the opinion that such an attempt would be somewhat premature.

In the meantime we believe that the discovery of specific events such as has been described for the pigeon [Carter and Drury, 1929] and is here described for the rat must prove important to the problem, and a study of such phenomena may be expected to prove of use in the characterisation of the constituents of the vitamin B complex.

Summary.

Deprivation of vitamin B complex leads to a severe bradycardia in young rats. The slow heart rate is due, not to the lowered food consumption but directly to the absence of the vitamin; for, (1) control animals on restricted amounts of complete synthetic diets fail to develop the bradycardia until moribund, (2) the bradycardia can be cured rapidly in the entire absence of food by injection or ingestion of vitamin B concentrates, and (3) prolonged restoration to normal heart rate can be effected by adequate provision of vitamin B, even when the food intake is restricted to the amount consumed in the last stages of the avitaminosis and the animal fails to make up lost weight. The curative substance is present in various vitamin B_1 concentrates tested and is comparatively readily destroyed by heat. It gives a response roughly graded to the dose administered. The same animal can be used for testing different specimens rapidly in turn, and it is suggested that the bradycardia may prove useful as an index in the course of fractionation of the components of the vitamin B complex.

The bradycardia is of sinus origin and is not due to vagal influence. It is not related to the experimental non-vagal bradycardia produced experimentally by the injection of muscle adenylic acid. Deficiency of vitamin A, or of vitamin D, or of A and D combined, has no significant influence upon the heart. Excess of vitamin D produces constantly a very slight bradycardia.

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CLXXX. VEGETABLE PROTEINS.
I. THE PROTEINS OF *DOLICHOS LAB LAB.*

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The field bean (*Dolichos lab lab*; Tamil name, Mochai; Kanarese, Avarai) is a legume which is widely cultivated in South India often as a mixed crop with cereals. The kernel of the seed enters into the diet of many South Indian households, and in the Mysore State the seeds are used as a delicacy when they are green for over four months in the year. The haulm, husk and pods are commonly used as fodder. As the kernel, which is widely used as an article of food and considered to be very nutritious, contains about 24 % of protein hitherto uninvestigated and as the quality of protein plays an important rôle in nutrition, the present work was undertaken.

Experimental.

The seeds used in this investigation belong to the cream-white variety raised in the Mysore State. They were sun-dried, after which the seed coats were removed in a huller and the clean kernel was ground to a fine powder (No. 40), which had the following composition: moisture 10·6 %, ash 2·95 %, ether-soluble extractives 1·57 %, protein 24·44 %, crude fibre 1·18 % and carbohydrate (by difference) 59·26 %.

Distribution of nitrogen in the meal. The nitrogen distribution in the meal was ascertained by extracting it successively with different solvents and the percentages of nitrogen extracted were as follows: water 72·8, 4 % sodium chloride 10·21, 70 % cold ethyl alcohol 1·27, 70 % hot ethyl alcohol 2·54, and 0·4 % sodium hydroxide 12·77; total nitrogen extracted 99·59 %.

All the nitrogen in the meal has been accounted for, and as is evident from the results recorded 72·8 % of the nitrogen is obtained in the aqueous extract, suggesting that probably the protein is the one usually known as "globulin." As this forms the major protein of the seed it formed the first object of study.

Influence of concentration of salt. 5 g. of the vacuum-dried meal were shaken for 3 hours with 50 cc. of salt solution of varying concentration; the extract was centrifuged at the end of that period and the nitrogen in the clear extract was estimated according to Kjeldahl. The results were: water 72·8 %,

5 % sodium chloride 71·57 % and 10 % sodium chloride 71·51 % of the total nitrogen, and indicate that water extracts more than salt solution. Probably the small quantity of electrolyte present in the meal is enough to peptise the protein.

Influence of the period of extraction. 5 g. lots of the vacuum-dried meal were extracted with 50 cc. of water for different periods. Determination of nitrogen in the centrifuged extracts gave the following percentages of the total: 0·5 hour 67·04, 1 hour 71·51, 2 hours 72·8, 3 hours 72·8, 4 hours *75·35*, 5 hours 71·51, and 6 hours 69·96.

Influence of the quantity of meal taken on the extraction. The great rôle played by the quantity of the solid phase in colloid solubility was clearly recognised by Hardy [1905] and Mellanby [1905] who found that the solubility of globulins in dilute solutions of electrolytes depended on the quantity of the solid phase. The quantity of globulin going into solution for the same volume of peptiser increases with increasing quantity of the solid phase although some of the globulin is left. Their experiments clearly bring out the difference between the solubility of molecularly disperse substances and that of colloids. While a molecularly disperse substance has one solubility for a given temperature and given solvent independent of the solid phase, the peptisation of colloids is not similar. The theoretical aspects of the question have been discussed by Ostwald [1927]. Similar behaviour was noticed by the present authors [1929] with the enzyme tyrosinase. The results of experiments to investigate this are given in Table I.

Table I. *The meal was extracted with 50 cc. of water.*

Weight of meal taken in g.	Nitrogen extracted (% of total)
5·0	72·8
7·5	73·22
10·0	75·02
12·5	**81·93**
15·0	75·8

It will be seen that the quantity of nitrogen extracted increases with the quantity of the solid phase, the maximum being attained when four parts of solvent are used for one part of meal.

Preparation of dolichosin.

It was pointed out by Narayanamurti and Ramaswami [1929] that aqueous extracts of the meal turn dark especially in the presence of air, owing to the action of the enzyme tyrosinase. As the action of this enzyme will essentially change the character of the protein with regard to its physical and chemical characteristics the usual methods of preparation of globulins are of no use. To prevent tyrosinase action smaller quantities of meal were extracted with larger quantities of water. 100 g. of the meal were extracted with 1000 cc. of distilled water in a glass-stoppered bottle of about 1200 cc. capacity, the

extract was quickly centrifuged and transferred to a specially constructed electro-dialyser shown in Fig. 1. The electro-dialyser consists essentially of two

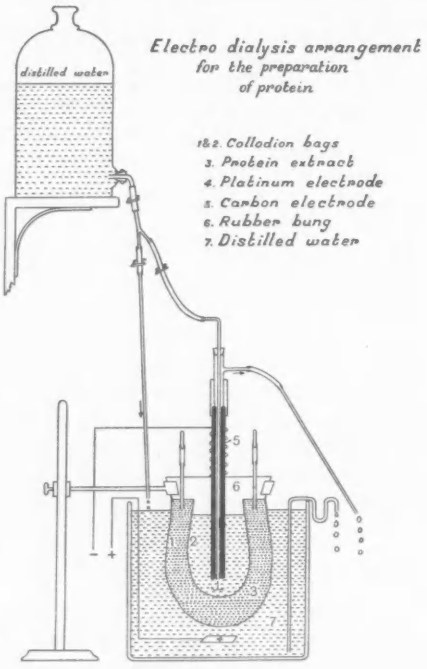

Fig. 1.

collodion thimbles. The outer one holds the protein extract, and distilled water is circulated through the inner one, the whole apparatus being immersed in a beaker of distilled water. A hole bored through the side of the carbon perpendicular to its axis allows water to pass out through the interspace between the wall of the glass tube and the wall of the hole bored through the length of the carbon. Thus continuous circulation of water is effected. By means of a constant level syphon arrangement continuous flow of water in the beaker is effected. The electro-dialysis was started at about 5 v. Enough resistance was introduced into the circuit to ensure a low current passing through. After a few hours the E.M.F. was increased to 10 v. and gradually raised to 30 v. In the course of about 40 hours all the protein had precipitated and the clear liquid above the precipitate was heated to see whether any protein not precipitated could be coagulated. No coagulum was formed. The precipitate was quickly filtered off on a Zsigmondy membrane filter and dried in a vacuum desiccator. As will be seen from the diagram the electro-dialysis was done out of contact with air, the collodion thimble holding the protein extract being

completely filled. Thus tyrosinase action was avoided. The protein was finely powdered and subjected to analysis.

Elementary composition of dolichosin. The results are given in Table II.

Table II. *Percentages on moisture and ash-free basis.*

	I	II	Average
Nitrogen	15·5	15·5	15·5
Carbon	52·0	51·66	51·83
Hydrogen	8·29	8·73	8·51
Sulphur	0·27	0·27	0·27
Phosphorus	0·55	0·55	0·55
Oxygen (by diff.)	23·39	23·29	23·34

Moisture in the protein preparation was 2·71 % and ash 0·66 %. All the sulphur is accounted for as cystine. The nature of the combination of phosphorus in dolichosin is being studied.

Nitrogen distribution in dolichosin. 3 g. of the protein were boiled with 15 cc. of 25 % hydrochloric acid till the reaction mixture no longer gave the biuret reaction. The hydrolysate was analysed according to van Slyke, advantage being taken of all the improvements suggested by Plimmer and Rosedale [1925], Daft [1929] and Linderstrøm-Lang [1927–9]. The results are given in the first column of Table III, the remaining columns give similar figures for proteins of allied species.

Table III.

	Globulin from			
Form of nitrogen	*Dolichos lub lab*	*Dolichos* biflorus*	*Cajanus† Indicus*	*Cicer* aretinum*
Amide	10·49	10·43	10·31	10·42
Humin	1·33	1·36	1·20	0·69
Basic:				
Arginine	16·84	14·45	11·37	19·31
Histidine	1·50	1·44	5·00	1·51
Lysine	8·20	9·25	8·20	8·48
Cystine	0·79	0·43	0·31	0·25
Non-basic:				
Amino	56·35	61·54	62·17	58·23
Non-amino	3·77	1·75	1·90	0·47
Total	99·27	100·65	100·46	99·36

* Narayana. † Sundaram, Norris and Subrahmanyan [1929].

In Table IV are given figures for the amounts of those amino-acids considered to be essential dietary constituents, which are contained in the globulins of *Dolichos* and allied species.

In common with other globulins dolichosin is characterised by a high percentage of basic nitrogen. As regards cystine content it compares favourably with the other globulins, and as regards tryptophan content it is essentially superior to the other globulins. Therefore it is evident that dolichosin is essentially superior to the other globulins and should be of high nutritive value.

Table IV. *Percentages of the essential amino-acids in dolichosin and other globulins as determined by different methods.*

	Globulin from				
Amino-acid	*Dolichos lab lab*	*Dolichos biflorus*	*Cajanus Indicus*	*Cicer aretinum*	Method of determination
Arginine	8·11	7·11	5·72	12·09	Van Slyke
Histidine	0·86	0·84	2·99	0·99	,,
Lysine	6·63	7·64	6·93	7·57	,,
Cystine	1·02	1·81	1·41	0·88	Folin and Looney [1922]
,,	0·96	—	1·39	—	Sullivan [1927]
Tryptophan	2·46	—	—	—	Folin and Looney [1922]
,,	2·59	—	0·12	—	Tillmans, Hirsch and Stoppel [1928]
,,	—	Present	—	0·41	Komm [1926]
Tyrosine	5·68	6·08	—	4·90	Zuwerkalow [1926]
,,	4·86	—	3·16	—	Tillmans, Hirsch and Stoppel [1928]
,,	4·93	—	—	—	Folin and Looney [1922]

Unlike cajanin and the other globulins it can be said to constitute a complete protein food.

The nitrogen distribution of dolichosin indicates that it differs from the protein isolated from *Dolichos biflorus*, principally in the tryptophan and cystine contents.

Summary.

The chief protein of *Dolichos lab lab* constitutes nearly 80 % of the nitrogen in the meal and is obtained in the aqueous extract.

In order to avoid tyrosinase action the protein has been prepared by subjecting the extract to electro-dialysis in a suitably constructed apparatus.

The nitrogen distribution in the protein has been determined and the nutritionally essential amino-acids have been estimated.

The nutritional value of dolichosin, as deduced from its composition, has been shown to be superior to that of the other globulins studied.

One of us (D. N.) is indebted to the Senate of the Indian Institute of Science for the award of a Research Studentship which enabled him to take part in this investigation.

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CLXXXI. IRRADIATION OF *DOLICHOS* TYROSINASE.

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Irradiation of enzyme solutions has been studied by several investigators, and excepting in a few isolated instances inactivation has been observed to follow this treatment. The interesting observation made by Narayanamurti and Ramaswami [1929] that tyrosinase from the field bean *Dolichos lab lab* is activated by exposure to the rays from a quartz mercury arc led us to undertake the present study.

Experimental.

Enzyme preparation. The enzyme was prepared by extracting 200 g. of the finely ground meal of the ungerminated seed of *Dolichos lab lab* with 600 cc. of distilled water in presence of toluene for three days at room temperature. After filtering through paper pulp the extract (containing a large quantity of protein) was dialysed under slight pressure in collodion bags against flowing distilled water for a week when all the proteins were precipitated and the solution was then centrifuged to remove suspended matter, the resulting brown liquid being clear. The enzyme thus prepared is very stable in aqueous solution for many weeks at 0°. As source of ultra-violet rays two lamps have been used in this investigation. A 110 v. D.C. atmospheric type lamp was used in the earlier experiments. A Hanovia "Homesun" 220 v. vacuum type arc was used in the later experiments. Both gave accelerations. The distance of the silica flask containing the enzyme solution was 15 cm. from the lamp and irradiation was done in presence of air. The experimental arrangement was very similar to that described by Narayanamurti and Ramaswami [1929] and the activity was estimated by the method of Raper and Wormall [1923].

Irradiation of dialysed extracts. The effect of exposing the dialysed extracts as such to the rays was first investigated. The results taken from the previous paper by Narayanamurti and Ramaswami [1929] are given in Table I.

Fresh experiments were conducted with two different extracts: (*A*) 3 or 4 months old, dialysed for over a month and colourless, (*B*) fresh and dialysed for 10 days. The effect of having a water-jacket of silica to absorb the heat rays

was also tried. In all cases acceleration was observed. The results are given in Table II.

Table I.

Time of exposure in minutes	Activity (cc. thiosulphate required)	Increase
	Fresh extract.	
0	4·4	—
5	8·0	3·6
15	8·9	4·5
30	9·2	4·8
90	9·4	5·0
	Old extract.	
0	7·1	—
5	8·7	1·6
30	10·9	3·8
60	10·1	3·0
180	9·8	2·7

Table II. *The reaction mixture was composed of 10 cc. of Walpole's acetate buffer of* p_H *6·5, 40 cc. of 0·05 % tyrosine solution, 5 cc. of water, 5 cc. of enzyme extract, and 10 cc. of toluene. The temperature was 30°. In this and following tables activity is expressed as cc. of thiosulphate required.*

	Old extract					
			Water jacketed		Fresh extract	
Time of exposure in min.	Activity	Increase	Activity	Increase	Activity	Increase
0	1·5	—	—	—	2·2	—
5	4·2	2·7	2·4	0·9	4·3	2·1
15	2·8	1·3	2·2	0·7	5·8	2·6

It is clear that the enzyme is activated whether fresh or old, water-jacketed or not. However, in the case of old extracts the increase in activity diminishes on continued exposure as observed in earlier experiments.

Influence of the container. The next point to be investigated was the influence of the container. The enzyme solution contained in silica and glass flasks of similar size and shape was irradiated with a Hanovia "Homesun," 220 v. D.C. 1·0 amp. normal current vacuum type lamp at a distance of 15 cm. The lamp was started 5 minutes prior to irradiation. The results are recorded in Table III.

Table III. *The reaction mixture contained 50 cc. of 0·06 % tyrosine solution, 5 cc. of water, 10 cc. of enzyme extract and 10 cc. of toluene. The temperature was 30° and the duration of reaction 3 hours.*

Time of exposure in min.	Activity		Increase in activity	
	Glass	Silica	Glass	Silica
0	1·9	1·9	—	—
5	2·0	4·5	0·1	2·6
15	3·5	5·0	1·6	3·1

It is seen that in the case of glass acceleration is still caused but to a much smaller extent than in the case of silica.

Effect of addition of irradiated water. The influence of irradiated water was tried (Table IV).

Table IV. *The reaction mixture was composed of 50 cc. of 0·06 % tyrosine solution, 5 cc. of water and 10 cc. of enzyme extract, 10 cc. of toluene being added. Temperature was 30° and duration of reaction 3 hours.*

Time of exposure of water in min.	Activity	Increase
0	1·9	—
15	2·6	0·7

Addition of irradiated water causes less marked acceleration than irradiation of the reaction mixture in glass vessels.

Effect of period of exposure. It was shown in the older experiments that activation depended on the time of exposure and that the increase in activity diminished with longer exposure in the case of old solutions. Some new experiments with old solutions are given in Table V.

Table V. *The reaction mixture contained 50 cc. of 0·06 % tyrosine solution, 10 cc. of enzyme extract and 10 cc. of toluene. Temperature was 31° and duration 4 hours.*

Time of exposure in min.	Activity	Increase
0	1·0	—
5	1·6	0·6
10	3·4	2·4
20	1·2	0·2
30	0·8	−0·2
60	3·6	2·6
75	4·2	3·2
90	3·4	2·4

Effect of keeping the irradiated enzyme on its activity. The next point to be investigated was the effect on its activity of keeping the irradiated enzyme. A quantity of the enzyme was irradiated with the help of a Hanovia "Homesun" at a distance of 15 cm. for 15 minutes and the activity of the enzyme was determined immediately and after several periods of exposure. The results are given in Table VI.

Table VI. *The reaction mixture was composed of 20 cc. of 0·06 % tyrosine solution, 10 cc. of enzyme solution and 5 cc. of toluene. The temperature of the reaction was 35° and duration 3 hours.*

Description of enzyme	Activity	Increase
Unexposed	6·9	—
Immediately after exposure	9·9	3·0
5 minutes after exposure	9·7	2·8
15 minutes after exposure	8·9	2·0
30 minutes after exposure	8·6	1·7
60 minutes after exposure	8·2	1·3

It is clear that the activation decreases on keeping. Whether this inactivation is due to destruction of an activator produced by irradiation or to increased lability of the activated enzyme only future experiments can decide.

Mechanism of the activation process. In a previous paper it was suggested that the activation might be related to the electro-kinetic potential of the enzyme particle. It was shown that the optimum p_H of the enzyme lies near its isoelectric point. It was therefore suggested that on exposure to ultraviolet light the cataphoretic mobility of the enzyme particle might be reduced. To test this assumption samples of the enzyme (old extract) exposed to two different periods of irradiation were subjected to cataphoresis and after 18 hours the liquids in the two arms of the apparatus were examined for their activities. The results are given below.

Table VII.

Time of exposure in min.	Activity	Increase in activity
0	1·0	—
5	1·6	0·6
30	0·8	−0·2

Description of the enzyme	Activity
Positive pole—unexposed	2·2
Negative pole—unexposed	4·8
Positive pole—5 minutes' exposure	4·4
Negative pole—5 minutes' exposure	5·0
Positive pole—30 minutes' exposure	3·8
Negative pole—30 minutes' exposure	6·8

It is clear from the results recorded that the enzyme is most active when it is least charged. On longer exposure the positive charge on the enzyme particle is increased thus causing a slight diminution in activity.

One of us (D. N.) is indebted to the Senate of the Indian Institute of Science for the award of a Research Studentship which enabled him to take part in this investigation.

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CLXXXII. FURTHER OBSERVATIONS UPON THE CORRELATION BETWEEN THE ARGININE AND VITAMIN B CONTENT OF DIETS, AND THE EFFECT OF THYROXINE UPON TUMOUR GROWTH.

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(*Received September 11th, 1930.*)

In an earlier communication reference was made to the difficulty of finding a diet for mice in which there would be a natural deficiency of arginine, but not of vitamin B [Gilroy, 1930, 3]. It had been shown previously [Gilroy, 1930, 1, 3], that arginine, administered by hypodermic injection, accelerated the growth of a transplantable tumour of the mouse (M 63), and it therefore appeared that a diet in which there was a relative deficiency of this amino-acid should have a depressing effect upon the rate of tumour growth.

Making use of Sakaguchi's [1915] reaction for combined guanidine, a comparison was made of the arginine content of various cereals, and it was found that the diet which most nearly fulfilled this requirement consisted of a proprietary preparation of barley, with rice starch, lettuce, and cod-liver oil. The probability that this diet lacked amino-acids other than arginine, and also vitamin B, made it desirable to include in any investigation of its effects upon tumour growth, unimplanted as well as tumour-bearing mice, in order that the former could be used to examine the efficacy of any additions to the diet in correcting a deficiency if loss of weight occurred. Synthetic diets were avoided, as it had been noticed that animals seldom eat such food in sufficient amount, and it was hoped that this important variable might be eliminated by using a diet which would be consumed in quantities comparable with those eaten by the controls. It was the object of this investigation to compare the growth rate of tumours in mice kept on this diet with that of tumours whose hosts were given standard food, and secondly to examine further the effect of thyroxine upon the rate of tumour growth [Gilroy, 1930, 2]. It seemed possible that animals receiving thyroxine might compensate for an augmented metabolism by consuming more food than untreated controls, and that its effect upon tumour growth was essentially a problem of nutrition. In the previous series of experiments all the mice received approximately equal rations and it

was proposed in the present investigation to give each group as much as they would eat, and to observe the actual quantity consumed at different stages of the experiment.

The technique and material were similar to those previously employed [Gilroy, 1930]. The rice starch was supplied by Messrs British Drug Houses, Ltd., and Engelberg's cod-liver oil was used in the special diet.

Exp. 1. 162 mice were implanted from four tumours, and distributed into sections so that each section included only implants from one tumour; the sections were then subdivided into groups for dietetic and other treatment, as follows:

Diet	No. of mice
1. Barley, rice starch, lettuce, cod-liver oil	27 males 11 females
2. Ditto + injections of thyroxine, 0·1 cc. of 0·1 % solution daily, on and after day 10	25 males
3. Brown bread, flaked maize, and lettuce	38 males 14 females
4. Ditto + injections of thyroxine as in group 2	37 males 10 females

The mice were not placed on special diet until six days after implantation. The result of this experiment is shown in Table I.

Table I.

The figures denote cross-sectional surface area of tumours in mm.² (group average). Death of a mouse is indicated by the sign +.

		Days since implantation						
Group	% takes	10	13	16	19	22	25	28
1	76·5	8·6	15·5 ++	30·3	40·7	61·6 ++	74·5	81·4
2	80·0	10·6	21·5	35·8 +	52·3 +++	59·0 +++	68·7 +	45·1 +++
3	79·0	12·3	30·9	65·9	94·9	135·0 ++	183·9 +	240·9
4	85·0	24·4	40·6	62·3	91·6	140·8 +++	175·7 ++++	236·9

The percentage of takes was not affected, as an interval of 6 days elapsed between the date of implantation and the exhibition of a special diet, but on the 10th day the average size of tumours in groups 1 and 2 was less than that of groups 3 and 4, a difference which may be attributed to diet. Before the commencement of treatment with thyroxine on the 10th day the mice in groups 3 and 4 were re-distributed in order that those bearing the largest tumours on that date should be allocated to group 4 which was to receive thyroxine. Previous experience had indicated that very large tumours did not yield to treatment and this point required confirmation; it also appeared that the inclusion of mice bearing large tumours in the control group must give the latter an initial advantage. The difference in average size of tumours in groups 3 and 4 was not therefore due to any treatment. The mice were weighed in

groups every 3 days when measurements were taken of the tumours, and the results are shown in Table II.

Table II.

	No. of mice		Weight of mice		Average weight		% loss or gain
	Day 7	Day 28	Day 7	Day 28	Day 7	Day 28	
Special diet; no injections	8	7	155·0	107·5	17·2	14·5	−15·7
	10	6	165·0	82·5			
	9	6	166·0	100·5			
	11	9	166·0	116·5			
Special diet + thyroxine	7	2	120·0	24·0	17·7	12·6	−28·8
	8	3	140·0	41·5			
	10	5	182·0	60·5			
Normal diet; no injections	13	13	224·0	254·0	16·0	20·7	+29·4
	9	8	161·5	169·5			
	16	14	213·0	251·5			
	14	13	235·5	276·5			
Normal diet + thyroxine	17	12	308·0	315·0	19·5	25·1	+28·8
	20	19	396·5	484·0			
	10	9	186·0	206·5			

It appeared to be of interest to compare these figures with the data obtained in previous experiments [Gilroy, 1930, 2], in which thyroxine and arginine were injected separately or together, the mice all receiving the same quantity and quality of food. The weights of these mice are shown in Table III.

Table III.

Injections	No. of mice in group	Weight of mice		Average weight		% loss or gain
		Day 10	Day 22	Day 10	Day 22	
Thyroxine daily	17	378·1	397·9	22·2	23·4	+ 5·4
Arginine substituted on 6th day	16	390·9	439·2	24·4	27·4	+12·6
Arginine and thyroxine daily	26	559·4	638·8	21·5	24·5	+13·9
Thyroxine daily and arginine on alternate days	6	125·3	132·5	20·9	22·1	+ 5·8
No injections, similar diet	26	557·3	627·4	21·4	24·1	+12·6

The diet which caused such rapid loss of weight in groups 1 and 2 of Exp. 1 was also given to 40 unimplanted mice, a further group of 12 mice being given for comparison a diet deficient in vitamin B. The mice were divided into groups and treated as follows:

Exp. 2.

Group 1. 12 adult males.
Diet as in exp. 1, groups 1 and 2.
Arginine injected (0·3 cc. of a 10 % solution daily) 9th–15th day.
Gelatin added to diet on day 15.

Group 2. 12 adult females.
Diet as in group 1.
Gelatin added to diet on day 15.

Group 3. 16 immature males.
Diet as in group 1.
Raw wheat germ* added to diet on day 15.

Group 4. 12 adult males.
Vitamin B-deficient diet (Glaxo).
Wheat germ added to diet on day 15.

* The wheat germ was the gift of Messrs Daren.

The result of this experiment is shown in Table IV, and Fig. 2.

Table IV.

	Group 1		Group 2		Group 3		Group 4	
Day	Av. wt.	% loss	Av. wt.	% loss	Av. wt.	% loss	Av. wt.	% loss
1	21·4	—	19·0	—	9·4	—	22·0	—
3	20·3	− 5·1	17·9	− 5·8	9·8	+ 4·2	19·5	−11·4
5	19·0	−11·2	16·2	−14·7	8·8	− 6·4	19·9	− 9·5
7	19·0	−11·2	16·1	−15·2	8·3	−11·7	19·6	−10·9
9	18·2	−14·9	15·3	−19·5	8·1	−13·8	19·4	−11·8
11	17·7	−17·3	14·7	−22·6	8·1	−13·8	18·8	−14·5
13	17·5	−18·6	14·7	−22·6	8·0	−14·9	17·8	−19·1
15	16·0	−24·0	15·2*	−20·0	8·2*	−12·8	17·2	−21·8
17	14·8*	−30·6	15·9*	−16·6	8·5	− 9·6	17·1	−25·2
19	14·1*	−34·0	17·2**	− 9·5	8·4	−10·6	18·8	−14·5
21	14·5	−32·2	17·3	− 8·9	8·7**	− 7·5	20·0	− 9·1
23	14·0	−34·5	16·8	−11·6	9·0	− 4·3	—	—

* Signifies a death.

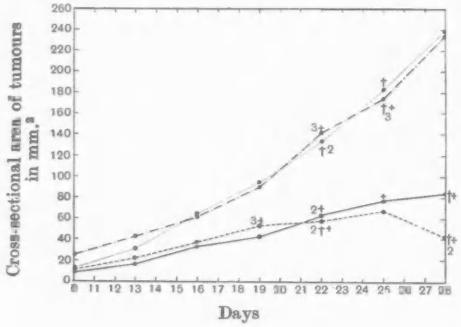

Fig. 1.

+ Death of mouse bearing small tumour. † Death of mouse bearing large tumour.
Group 1. ———. Group 2. - - - - - -. Group 3. ..... Group 4. -·-·-·-.

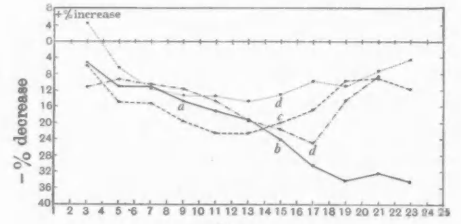

Fig. 2.

——— Adult ♂ } Arginine-
- - - - - Adult ♀ } deficient
..... Immature } diet.
-·-·-·- Adult ♂ Vitamin B-deficient diet (Glaxo).

Arginine injections {Begun (*a*). Stopped and gelatin added to diet (*b*).
Gelatin added to diet (*c*).
Wheat germ added to diet (*d*).

Discussion.

It is evident that the experimental diet was not deficient in arginine only, since injections of this amino-acid increased rather than checked the loss of weight (group 1), and the subsequent addition of gelatin to the diet produced no improvement in this group and only a temporary weight increase in group 2. From this it appeared that the diet lacked some important constituent other than an amino-acid, it having been shown by Downey [1924] that gelatin is supplementary to pearl barley in this respect. The amount of food consumed was at first quite comparable with that of controls on normal diet, the appetite of the mice only failing when a considerable loss of weight had already occurred. After the addition of gelatin to the diet the food consumption again rose for a few days, and this may perhaps account for the temporary improvement in group 2, in which the average weight at this time was further increased by the death of three of the smallest mice.

The addition of wheat germ to the diet produced more permanent results: it is possible that this corrected a deficiency in tyrosine or tryptophan which would not be supplied by gelatin[1] [Mitchell and Hamilton, 1929]. The barley used in the experimental diet gave a negative result when tested for these amino-acids, using the Adamkiewicz, Liebermann, Millon and xanthoproteic tests, whereas the wheat germ gave a positive reaction to the last of these. It had been found in earlier experiments that male mice on a standard vitamin B-deficient diet injected with arginine lost weight more rapidly than did uninjected mice [Gilroy, 1930], and it is to be noted that in the present experiment also injected males showed a greater loss of weight, and subsequently failed to respond to dietary additions which benefited the uninjected mice. The diet included lettuce, which would contain water-soluble accessory food factors, but it was shown by Sherman [1929], that lettuce is not growth-promoting even in large amounts. From these considerations it seems probable that the introduction of vitamin B in the wheat germ was the deciding factor, rather than the correction by the same means of an amino-acid deficiency; in this case the suggestion would be justified that diets which are naturally deficient in arginine are also deficient in vitamin B. The inhibition of tumour growth in mice on this diet was similar to that induced by avitaminosis [Gilroy, 1930, 3], but this result is of no significance as it would probably accompany a state of severe malnutrition, whatever its cause might be. The mortality from thyroxine on this diet was very considerable; in one group it was so high that the data on tumour growth could not be utilised at all.

An analysis of the weights of implanted mice subsequently injected with thyroxine shows that increase in body weight is in general parallel with, and probably dependent in part upon, increase in tumour size. The effect of thyroxine upon tumour growth as shown by body weight could be modified

[1] Millon's reaction was positive for the sample of gelatin used (prepared by Messrs J. and G. Cox, Ltd.).

by injection of arginine which appeared to compensate for the effect of thyroxine partially or completely according to the amount administered (Table III); daily injections of arginine or the substitution of the latter for thyroxine halfway through the experimental period balanced the increased catabolism and gave values for weight corresponding with those of untreated controls. In the present experiment, the results of which are shown in Table II, the percentage increase in weight of mice on normal diet hardly differed in mice injected with thyroxine and in untreated controls, although arginine was not exhibited to counter its effects. The explanation of this apparently anomalous result is to be found in the larger quantity of food consumed by treated mice, which, 2–5 days after injections of thyroxine were commenced, developed an appetite far greater than that of the controls. It therefore appears that the injection of arginine or the assimilation of a greater quantity of food is equally effectual in preventing the tissue loss which thyroxine would otherwise induce. The administration of a diet rich in carbohydrate is known to counteract the destruction of tissue protein which would result from inanition [Mitchell and Hamilton, 1929], and a diet of brown bread and maize, if eaten in sufficient quantities, might be expected to exert a similar effect.

Although the curves of tumour growth shown in Fig. 1 appear to approximate in both groups on normal diet, the increase in tumour size in mice receiving thyroxine was in reality considerably less than in the untreated controls. In the group injected with thyroxine 6 mice bearing small tumours (*i.e.* not exceeding 120 mm.^{2}) died in the last 6 days of the experiment, while only one mouse bearing a large tumour died. In the untreated group, on the other hand, 3 mice with very large tumours died during the same period and none died in which the tumours were small. A consideration of these facts will show the average weight and tumour size to have been less in mice receiving thyroxine, and greater in untreated mice at this stage of the experiment if mortality were taken into account, and that the curves from the 22nd to the 28th day therefore give an erroneous impression of the relative rate of growth.

In previous experiments thyroxine had not injured the health of the mice, and no deaths had occurred in the animals receiving it. The present experiment was continued for 18 days instead of 12 as before, and this may account for the higher mortality rate in injected mice. It is perhaps significant that of the 7 mice which died as a result of treatment only one had a large tumour, and this was originally much above average size (153 mm.^{2} on the 10th day of the experiment). It may be inferred that in these a smaller dose would have sufficed, inducing in such individuals a response equivalent to that obtained by the dosage actually adopted in the case of a large number of mice, in which tumour growth was checked without serious detriment to the host. Similarly mice in which the rate of tumour growth was rapid might have tolerated a larger dose, as these individuals were evidently resistant to its effects. It is clearly impossible to disregard individual variations and yet produce in every animal just that degree of response which will prevent the further growth

of tumours without serious injury to the host. It seems possible that those individuals in which a response is obtained with relative ease are those in which a greater potential activity of the thyroid exists, and that this represents a diathesis which is inimical to the development of neoplastic growth.

Summary.

Fifty-two male mice were implanted with tumours (M 63), and 5 days later were placed on a diet deficient in arginine. On this diet tumour growth was inhibited to an extent similar to that induced by a diet deficient in vitamin B. This was accompanied by steady loss of weight and a high mortality rate in 25 mice which were also injected with thyroxine.

Forty unimplanted mice were placed on a similar diet, and 12 mice were given for comparison a diet deficient in vitamin B. The addition of gelatin on the 9th day caused only a temporary recovery, while wheat germ induced a permanent increase in weight. Injections of arginine increased the loss of weight, a phenomenon only associated previously with a deficiency in vitamin B.

Seventy-five male mice implanted with tumours were injected with thyroxine daily from the 10th to the 28th day after implantation. A slight but definite inhibitory effect upon tumour growth was observed in spite of an increase in weight, the less marked effect in this second experiment being due to the larger quantity of food consumed by mice receiving thyroxine as compared with untreated controls. The results were comparable with those obtained when arginine was injected during the period when the mice received thyroxine in an earlier series of experiments.

The expenses of this work were defrayed by a grant from the Medical Research Council, to whom I wish to express my thanks. I am also indebted to Prof. Crew for scientific hospitality, and for his criticism and advice.

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CLXXXIII. ON THE ORGANIC MATTER GIVEN OFF BY ALGAE.

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According to the hypothesis first proposed and maintained by Pütter [1909], aquatic animals of all groups up to and including fishes live largely on the organic substances found dissolved in natural waters. These substances are taken to be mainly derived from the photosynthetic processes of the plankton algae, it being assumed that the products of these processes become fixed in the bodies of the algae only to a slight extent, and are mainly given off to the surrounding water.

One of us [Krogh, 1930, 1] has described methods for a fairly accurate determination by combustion of organic substances dissolved in fresh water. These methods utilise the fact that organic substances belonging to the classes of proteins, fats and carbohydrates (as well as several others), can be oxidised by combustion in nitrogen by means of cupric oxide, liberating CO_2 and N_2 as gases. If a measured volume of oxygen be added, while the mixture is still at a red heat, the reduced copper is again oxidised and it is possible by gas analysis to determine the carbon dioxide and nitrogen liberated and the residue of oxygen, left over after the oxidation. The amount of substance suitable for such a determination in its micro-form is 3–15 mg., and the volume of water supposed to contain a quantity between these limits is concentrated to a small bulk; certain ions (Mg and Ca) which interfere with the combustion are removed and the solution is finally evaporated to dryness in the combustion tube. For the technical details the reader is referred to the paper quoted above.

In a separate paper [Krogh, 1930, 2] experiments on the absorption of dissolved organic substances by animals are described and here we shall deal with the alleged liberation of such substances by algae. Our experiments fall into two groups. In one series we have worked on pure cultures of *Scenedesmus obliquus* in artificial media, while in a second we have taken natural water from a lake and brought the algae to a luxuriant growth.

Scenedesmus *experiments*. We are greatly indebted to Prof. Chodat of Geneva, who supplied us with pure cultures of the algae *Scenedesmus obliquus*, *Stichococcus bacillaris*, *Chlorella vulgaris*, and *Chlorella rubescens*, and gave us

many useful hints regarding their cultivation. After a number of trials we selected *Scenedesmus* as the form showing the best rate of growth under the conditions imposed by the limitations of our combustion analysis. We could not use nitrate as a source of nitrogen, because it would give rise to error in the combustion, and had therefore to experiment with urea and ammonia. Urea can be retained in the final evaporation only if the temperature is kept below 70°, while ammonia can be driven off entirely at p_H 9·5. In our earlier experiments we did not drive off the ammonia but determined it in the acid concentrate before the final evaporation.

Our final substrate was made up by diluting a mixture of 1 cc. of *M* $CaCl_2$, 0·15 cc. of *M* $MgCl_2$, 0·3 cc. of *M* KH_2PO_4, 0·05 cc. of *M* Na_2HPO_4, and 0·01 cc. of *M* Fe_2Cl_6 to 1 litre with water re-distilled from hard glass and was distributed in suitable flasks with 250 cc. in each and autoclaved for 1 hour at 125°. After the autoclaving the solution was sometimes a little cloudy but could be cleared with a few drops of *M* HCl. The reaction was brought to the optimum of p_H 8·4 by the addition of sterile *M* NaOH, about 0·1 cc. being required for each bottle. A molar solution of $(NH_4)_2CO_3$ was sterilised by filtration and a suitable amount, usually 0·15 cc., was added to each flask. To insure a rapid growth the bottles were usually inoculated with 1–2 cc. of an older culture.

The culture flasks plugged with cotton wool were placed in a white box, exposed to strong artificial light (two 75 c.p. reflector lamps at a distance of about 30 cm. above the flasks) and incubated at a temperature of about 25° for 18–45 days. To insure a liberal supply of CO_2 a slow current of the gas was constantly flowing into the box through a wash-bottle. At the end of the experiment the algae were filtered off by a membrane filter as described by Krogh [1930, 1] and the filtrate, usually from 2 flasks (500 cc.), was taken for analysis. The algae were quantitatively removed from the filter, dried, weighed, and in some cases analysed.

To the water, concentrated to about 20 cc., we added excess of ammonia and sufficient phosphoric acid to form $Ca_3(PO_4)_2$ and $MgNH_4PO_4$. After 24 hours the precipitate was filtered off and the excess of ammonia and CO_2 were driven off in a vacuum, as described by Krogh, before the fluid was finally evaporated down in the combustion tube. In some experiments we used a natrolite filter to get rid of calcium, magnesium and ammonia, but this proved less satisfactory.

Exp. 1.

Culture 15. ii. 29—1. iv. 29. Two flasks 500 cc. Weight of algae dried at 110° for 5 hours 80 mg.

Analysis of substrate after the culture (gas volume reduced to 770 mm. and 0°): CO_2 5·08 cc., O_2 4·45 cc., N_2 0·46 cc.

This analysis is not very reliable, the oxygen value being certainly too low and the nitrogen possibly a little too high. The pure substrate was not analysed but may very well have contained organic material corresponding to 2 cc. of CO_2. The result shows that the organic substance *possibly* given off to the water is at most a small fraction of that retained in the algae.

Exp. 2.

Culture 20. iv. 29—8. v. 29.

Two flasks 500 cc. Dry weight of algae 50 (–60) mg. CO_2 2·58 cc., O_2 3·25 cc., N_2 0·16 cc., $\frac{CO_2}{O_2}$ 0·80. Two other flasks: CO_2 1·35 cc., O_2 1·67 cc., N_2 0·10 cc., $\frac{CO_2}{O_2}$ 0·81. Dry weight of algae from 6 flasks 145 mg. Average for 2 flasks 48 mg. Filtrate 1500 cc. 500 cc. analysed after filtration through natrolite gave CO_2 2·25 cc., O_2 2·42 cc., N_2 0·38 cc., $\frac{CO_2}{O_2}$ 0·93.

This analysis is less reliable than the first two, since it was found later that the natrolite would give off traces of organic substances.

Analysis of dry algae per 10 mg.: CO_2 6·48 cc., O_2 8·18 cc., N_2 0·53 cc., $\frac{CO_2}{O_2}$ 0·79.

The substrate incubated without algae gave 1·17 cc. CO_2 and 0·17 cc. N_2. The oxygen determination was lost, but may be assumed as 0·7.

We find therefore that the algae in 1 litre gave off to the water organic substances yielding 2·76 cc. CO_2 and 0·09 cc. N_2 with a combustion quotient of about 0·8. In the organisms organic substance was stored to the extent of 96 mg. yielding 62 cc. CO_2 and 5·1 cc. N_2 with a quotient of 0·79.

Calculated as protein, carbohydrate, fat and gram-calories the figures are, for 1 litre:

	Protein mg.	Fat mg.	Carbohydrate mg.	Calories
Water	0·7	0·85	1·05	16·5
Algae	40·0	13·0	5·5	374·0

The total weight of the organic substances calculated for the algae

$$40 + 13 + 5{\cdot}5 = 58{\cdot}5 \text{ mg.}$$

falls far short of the 96 mg. dry weight and determinations of the ash content were therefore made on samples left over, but unfortunately not dated. The ash was found to be 30·3 % and 41·4 % respectively in two samples. The above calculation would leave 37·5 mg. or 39 % for the ash. The results show that in the conditions of the experiment these algae give off less than 5 % of the organic matter formed by photosynthesis to the water and store at least 95 % in their own cells.

Natural water experiments. Samples of water were taken from the surface of the lake Furesö on Nov. 28. A sample of this water was filtered through 30″ collodion filters as described by Krogh [1930, 1], which hold back all organisms from bacteria upwards, and 1 litre of the filtered water was concentrated, precipitated with NH_4OH and H_3PO_4, and filtered after standing for 24 hours in the ice chest. From the filtrate the NH_3 and later CO_2 were driven off and the fluid evaporated down in the combustion tube. It yielded 8·41 cc. CO_2, 0·50 cc. N_2 and used 8·58 cc. O_2.

Samples of the unfiltered water and of water filtered through paper were saturated with air at room temperature and put into 1 litre bottles. To each sample was added 0·05 cc. of *M* H_3PO_4 and 0·15 cc. of *M* NH_4OH. The stoppers were provided with wide tubes reaching down to the middle of the bottle and

through these 7 cc. samples were syphoned off at intervals for oxygen titrations. The amount of water removed was replaced from a small bottle treated in the same way and kept under the same conditions. The bottles were placed in a water-bath at a temperature of 23° ± 1° and exposed to strong artificial light. The water-bath was painted white on the inside. The oxygen determinations gave in cc. per litre:

	Filtered	Unfiltered
Nov. 30	5·95	5·93
Dec. 5	2·75	3·70
„ 12	22·8	17·55
„ 28	28·5	33·6
Jan. 10	23·9	24·0

During the latter part of the experiment much air was given off in bubbles.

The organisms present in the paper-filtered water were a species of *Nitzschia* and a species of *Chlorella*, which were both very common, while *Scenedesmus quadricauda* was less abundant. In the unfiltered water *Scenedesmus quadricauda* was absolutely dominant, a *Chlorella* was common and several other forms were present.

On Jan. 13 the samples were filtered through 30″ filters but most of the algae adhered to the glass and could not be recovered. The filtered samples were concentrated as before, evaporated and analysed with the following results:

	CO_2 cc.	O_2 cc.	N_2 cc.
Paper-filtered	11·40	12·03	0·53
Unfiltered	9·95	10·59	0·40
Original water	8·41	8·58	0·50
Increase filtered	3·0	3·45	0
Increase unfiltered	1·55	2·0	−0·1

In the same period the production of organic substance corresponds at least to the maximum increase in oxygen content observed, 25·75 cc. in the filtered and 29·9 cc. in the unfiltered water. In this case again the organic substance going into solution in the water cannot be more than 10 % of the amount produced and is probably lower because the animals and bacteria must have given off a certain amount of organic material during the 44 days period. The influence of the animals must be slight since the paper-filtered water, from which animals must have been removed, gives the largest increase in organic material.

In a second experiment quantitative determinations were made on the organism developing during the experimental period. Samples of water were taken from the Furesö on June 5 at 2 m. depth. One sample was filtered through filter paper and another through plankton gauze (No. 20) to remove larger organisms. Each filtered sample was taken through a 30″ collodion filter and put into 1 litre sterile flasks to which 0·05 cc. of *M* phosphoric acid and 0·15 cc. of *M* ammonia were added. The flasks were inoculated with plankton material from the collodion filters and cultivated in natural light at a window facing N.W. The sample inoculated with paper-passing material was cultivated in this way for 18 days and the sample inoculated with gauze-passing material

for 19 days. At the end of this period the plankton was filtered off, dried, weighed, and analysed, while the filtrate was subjected to the regular procedures leading up to a combustion analysis.

The sample inoculated with paper-filtered material gave the following results.

The organisms present were *Scenedesmus obliquus* Kg. (very abundant), *Ankistrodesmus Falcula* Brunnth. var. *tenellus* (abundant), *Ankistrodesmus Braunii* Lemm. var. *pusillus* Printz (common) and *Ankistrodesmus falcatus* Ralfs var. *spirilliformis* West (rare)[1].

The total dry weight of the algae in a 1000 cc. sample was 51·8 mg. with 22·6 % ash. The combustion gave per g. 726 cc. CO_2 and 43 cc. N_2 with an absorption of 919 cc. O_2 (R.Q. 0·79). Calculated as crude protein, fat and carbohydrate these figures give respectively 34·6, 20·3 and 13·8 %. The sum of the constituents as calculated is 68·7 leaving 31·3 % for ash and water. A sample dried further for 3 hours at a temperature of 100° lost 6·9 % in weight, leaving only 1·8 % not accounted for by the calculation. The combustion value of the material works out as 4400 cal. per g. or 228 cal. for the organisms in 1 litre.

The gauze-filtered sample gave as the absolutely dominating organism a species of *Chlamydomonas*, while a few individuals of *Ankistrodesmus falcatus* were present.

Dry weight 48·0 mg.

Ash, 2 determinations, 17·8 and 17·3 %, mean 17·7 %.

	cc. CO_2 per g.	cc. O_2 absorbed per g.	R.Q.	cc. N_2 per g.
Combustion (*a*)	698	848	0·82	36
(*b*)	691	841	0·82	42

The average corresponds with 305 mg. of crude protein, 237 mg. of carbohydrate and 140 mg. of fat, leaving 318 mg. for ash and water. Drying at 100° gave 9·8 % extra water and we have 45 mg. per g. not accounted for. The combustion value is 4100 cal. per g. or 197 cal. for the organisms in 1 litre.

The analysis of the water gave per litre:

	CO_2 cc.	O_2 cc.	N_2 cc.	R.Q.
Paper-filtered	13·17	14·47	0·65	0·91
Gauze-filtered	11·06	12·10	0*	0·915
Original water	8·29	9·12	0·44	0·91
Increase paper-filtered	4·88	5·35	0·21	0·91
Increase gauze-filtered	2·77	2·98	?*	0·93

* There must be an error in this result, but its source could not be detected. The apparatus was found to be airtight in a test performed just after the analysis.

Assuming a caloric value for the oxygen of 5·0 we have in the paper-filtered sample an increase in organic material corresponding to 27 cal. or 12 % of the combustion value of the plankton and in the gauze-filtered of 15 cal. or 8 % of the plankton.

[1] We are indebted to Mr G. Nygaard for the determination of the organisms in our samples. The variety *tenellus* of *A. Falcula* turns out to be new and will be described by him.

SUMMARY.

All the culture experiments agree in showing that the organic material synthesised by the assimilation of freshwater algae is almost quantitatively stored in the cells of the algae, while a fraction amounting at most to 10 % may possibly be lost to the surrounding water. We think it most probable that the organic substances directly lost during assimilation are wholly negligible, and that the losses observed in these experiments are mainly due to dead and decomposed organisms.

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CLXXXIV. THE BEHAVIOUR OF δ-KETO-*n*-HEXOIC ACID IN THE PERFUSED LIVER.

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In an investigation of the oxidation products produced from the ammonium salts of the saturated fatty acids by hydrogen peroxide it was shown by Clutterbuck and Raper [1925, 1] that γ- and δ-oxidation occurred, in addition to the β-oxidation which had been described previously by Dakin [1908, 1, 2, 3]. The γ- and δ-keto-acids produced in this way on further oxidation yield succinic acid, this change being more pronounced with the δ- than with the γ-keto-acids. This mode of oxidation of fatty acids may be of biological interest. Attempts have been made therefore to find out whether it occurs in the body. The results of the administration of β-phenyl-*n*-hexoic acid have already been described [Clutterbuck and Raper, 1925, 2]. If this acid underwent γ- or δ-oxidation and were further split between the γ- and δ-carbon atoms it should yield phenylsuccinic acid; the latter was however not found to occur. Since a large part of the β-phenylhexoic acid administered was recovered in the urine and was therefore apparently difficult to oxidise, the result was inconclusive. The experiments described in the present paper aimed at getting a more definite answer to the question as to whether succinic acid may be obtained by the oxidation of fatty acids in the body following δ-oxidation of the carbon chain. The sodium salt of δ-keto-*n*-hexoic acid has been subjected to liver perfusion with a view to determining whether it gives rise to acetoacetic acid or not. The following two modes of oxidation may be considered to represent the possible methods of breakdown.

(*a*) $CH_3.CO.CH_2.CH_2.CH_2.COOH \rightarrow CH_3.COOH + HOOC.CH_2.CH_2.COOH$
succinic acid

(*b*) $CH_3.CO.CH_2.CH_2.CH_2.COOH \rightarrow CH_3.CO.CH_2.CO.CH_2.COOH$
$\rightarrow CH_3.CO.CH_2.COOH$
acetoacetic acid

If scheme (*a*) is correct then perfusion of δ-keto-*n*-hexoic acid through the isolated liver should not give rise to the formation of acetoacetic acid, whereas under scheme (*b*) a considerable production of acetoacetic acid would be expected. It has also to be borne in mind that acetic acid under certain conditions

gives rise to acetoacetic acid in the liver, but, as will be shown presently, this even if produced under scheme (*a*) could not account for the results obtained.

The experiments described consist of a comparison of the yields of acetone substances produced by *n*-hexoic acid and δ-keto-*n*-hexoic acid under conditions as nearly alike as possible. Three experiments were carried out with each acid and it was found that the δ-keto-acid, although yielding somewhat less acetone and β-hydroxybutyric acid than *n*-hexoic acid, gave amounts which were so large that there seems to be no doubt that it undergoes oxidation according to scheme (*b*). These experiments therefore furnish no support for the view that δ-oxidation of fatty acids followed by scission between the γ- and δ-carbon atoms occurs in the animal body.

After the perfusion both the blood and the liver were examined for the presence of succinic acid. None was found in the blood but small amounts were obtained from the liver. The presence of succinic acid in the liver did not depend however on the nature of the acid undergoing oxidation, since it was found on all occasions whether *n*-hexoic or δ-keto-*n*-hexoic acid had been added to the perfusing blood or when no addition had been made. It may be pointed out that Magnus-Levy [1902] found succinic acid in the liver of dogs and other animals after autolysis. The amount was independent of the period of autolysis and it seems reasonable to believe that it may have been present in the fresh organ.

The possibility that the acetone substances obtained from δ-keto-*n*-hexoic acid might have been produced from acetic acid has been referred to. It has been shown by Loeb [1912] that when the liver is perfused with blood containing sodium acetate a definite but small formation of acetoacetic acid occurs. This we have confirmed. But using for the perfusion the same conditions as were used for δ-keto-*n*-hexoic acid, the amount of acetone substances produced was very much smaller than with the latter acid. We conclude therefore that the formation of acetone substances when δ-ketohexoic acid is oxidised in the liver must be due mainly to β-oxidation.

Experimental.

The δ-keto-*n*-hexoic acid was prepared as described by Clutterbuck and Raper [1925, 1]. When required 2 g. of the crystalline acid hydrate (equivalent to 1·76 g. acid) were dissolved in a small amount of water, neutralised with sodium hydroxide and added to the blood.

Perfusion.

The apparatus used for the perfusion was that described by Burn and Dale [1926] with the exception of the oxygenator. As originally described we found this to be inadequate when a dog's liver is used, although in our experience with a cat's liver, because of its smaller size, oxygenation of the blood has been sufficient. The modification we have introduced is illustrated by Fig. 1. In Burn and Dale's apparatus the blood after leaving the liver is led on to a

silver disc spinning rapidly at the top of a wide glass cylinder. This throws it on to the wall of the cylinder and it runs slowly to the bottom where it forms a reservoir. From this the blood is now drawn up to the pump which circulates it through a heating coil to the liver. Instead of depending for oxygenation on the contact of the film of blood on the wall of the cylinder with oxygen led into its interior as originally described by Burn and Dale, we have bubbled oxygen directly through the reservoir of blood at the bottom of the cylinder. For this purpose a Jena glass-filter (593/L7) in the shape of a thistle funnel was used. This produces a stream of extremely fine bubbles which form a compact froth on the surface of the blood. When a layer of froth about 10 cm. deep

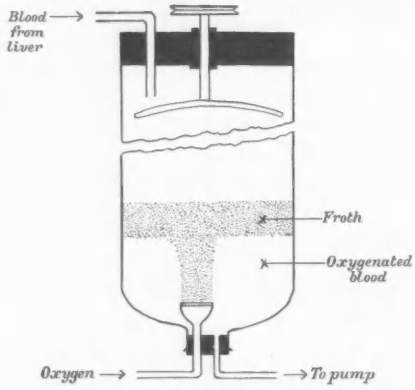

Fig. 1

has been formed the oxygen may be shut off until absorption or bursting of the bubbles has reduced the layer to about 5 cm. It should then be increased again to 10 cm. by further admission of oxygen. The blood percolating through this fine froth becomes completely oxygenated.

The dogs were anaesthetised with ether and bled from the abdominal aorta. Warmed Ringer's solution was introduced into the distal end of the divided aorta between the intervals of bleeding. In all about 400–500 cc. of blood diluted with Ringer's solution were usually obtained. The bile duct, hepatic artery, and tributaries of the portal vein were then ligatured and a cannula was inserted into the vein itself. The thorax was immediately opened, an incision was made into the right auricle and 1–2 litres of ice-cold normal saline were passed through the liver. The object of this was to cool the liver so that it did not suffer from oxygen lack during the subsequent part of the operation for its removal when it had no blood supply. Having cooled the liver in this way a cannula was inserted into the inferior vena cava above the diaphragm and the vessel was then ligatured in the abdomen just above the suprarenal glands. The liver was then removed and placed in the perfusion apparatus.

The organ was perfused for 75 min. after the temperature of the entering blood had reached 38°.

Analyses.

Shaffer's method [1908] was used for estimating acetone, acetoacetic acid and β-hydroxybutyric acid in the blood after perfusion. Schenk's reagent was used for precipitating the blood proteins. As a rule 50 cc. of blood were taken for the estimation. The presence of substances other than acetone which appear in the distillate when either acetone and acetoacetic acid or β-hydroxybutyric acid are being determined necessitated the treatment of these distillates by Masuda's method [1912]. The figures in Table I give the results obtained. The acetone and acetoacetic acid are reckoned as acetone in the whole of the blood which was used in the perfusion. The figures for β-hydroxybutyric acid represent the acid itself and not acetone in the total blood.

Table I.

Experiment	Addition to blood	Acetone + aceto-acetic acid mg.	β-Hydroxy-butyric acid mg.	Wt. of liver g.	Volume of blood used cc.	Glycogen content of liver
1	Nil.	Trace	Trace	225	490	Poor
2	Nil.	7·7	2·8	200	400	Poor
3	Nil.	1·5	9·5	200	400	Rich
4	2 g. *n*-hexoic acid as Na salt	4·1	166	225	400	Poor
5	2 g. *n*-hexoic acid as Na salt	4·6	215	180	400	Poor
6	2 g. *n*-hexoic acid as Na salt	22·5	242	165	485	Moderate
7	2 g. δ-ketohexoic acid hydrate as Na salt	22·6	65	450	465	Poor
8	2 g. δ-ketohexoic acid hydrate as Na salt	21·4	124	270	425	Poor
9	2 g. δ-ketohexoic acid hydrate as Na salt	59	143	225	565	Rich
10	2 g. sodium acetate	28·4	14·5	285	460	Rich

Further examination of the liver and blood. This was undertaken to estimate the amount of unchanged δ-keto-acid in the blood and liver after the perfusions with δ-keto-acid and also to find out whether succinic acid was present. An approximate method of estimating the δ-keto-acid by conversion into the semicarbazone was worked out and the percentage recovery by this method was determined when varying amounts of the acid were added either to whole blood or to a mixture of 2 parts of blood with 1 part of normal saline. The aqueous extract of liver or an aliquot part of the blood filtrate (after removing proteins by Schenk's reagent and mercury as sulphide) was neutralised, evaporated to small bulk, saturated with ammonium sulphate and filtered. The precipitate was washed with small amounts of saturated ammonium sulphate, and the united filtrate and washings were strongly acidified with syrupy phosphoric acid. This solution was extracted for 40 hours with ether in a continuous extractor. The ether was removed by distillation. The residue

was extracted with warm benzene, the benzene-insoluble residue being reserved for the detection of succinic acid as described later. After distilling off the benzene a few drops of water were added to the residue followed by solid semicarbazide hydrochloride and powdered sodium acetate. The semicarbazone of the keto-acid rapidly separated out. It was filtered off, washed with water, dried in a vacuum desiccator and weighed. The results shown in Table II were obtained when the amounts of δ-keto-acid stated were added to 100 cc. blood or the blood-saline mixture.

Table II.

Keto-acid added g.	Keto-acid recovered g.	% recovery
0·2195	0·1935	88
0·395	0·334	84
0·615	0·543	88
	Mean	86

In the three perfusion experiments (Nos. 7, 8 and 9) in which 2 g. of the keto-acid hydrate (equivalent to 1·76 g. keto-acid) were used the amounts of keto-acid in the whole blood at the end of the perfusion and assuming on the basis of the control experiments an 86 % recovery were, 0·41, 0·24 and 0·20 g. respectively. The amount recovered from the liver was smaller, namely 0·10, 0·035 and 0·023 g. so that a considerable amount of the acid had disappeared during the perfusion. The yield of acetone substances shows that some of the acid had undergone β-oxidation, but it is also probable that a portion had been reduced to δ-hydroxy-*n*-hexoic acid. This may account for the smaller yield of acetone substances compared with that obtained from *n*-hexoic acid under the same conditions.

That portion of the ether extract which was insoluble in benzene was neutralised with *N* NaOH and precipitated with 5 % silver nitrate. The silver precipitate was washed with a little water and rubbed up with 10 % sulphuric acid and the solution was then extracted five times with ether. The ether was removed by distillation and the crystalline residue washed with a small amount of ether and dried. The M.P. and the pine splinter reaction showed that it was succinic acid. In a control perfusion without any addition to the blood, and in those perfusions in which *n*-hexoic or δ-ketohexoic acid had been added, succinic acid was always found in the liver in small amount. On the other hand, none was found in the blood in the perfusions with δ-ketohexoic acid. The blood was not examined in the other experiments.

Summary.

1. The sodium salt of δ-ketohexoic acid on perfusion through the liver yields a considerable amount of acetone substances. This indicates that it undergoes β-oxidation and not further γ- or δ-oxidation.

2. Succinic acid was normally found in the liver in small amount. Its presence is not apparently related to any addition made to the perfusing blood.

3. An improved method of oxygenating the blood in perfusion experiments is described.

Our thanks are due to the Government Grant Committee of the Royal Society for a grant in aid of this work.

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CLXXXV. THE MALE HORMONE. IV[1].

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Until quite recently we have prepared the male hormone from urine acidified to Congo red and extracted with chloroform. The chloroform residue was steam-distilled and finally taken up with alkali. It was then observed that while steam distillation is an excellent procedure for getting rid of objectionable impurities, it also results in an appreciable loss in hormone activity. This was especially marked when superheated steam was used. Maximum activity was obtained only when the final residue could be completely dissolved. On many occasions this was not possible except by first treating the residue with *N* alkali and then with *N*/20 alkali. When these two separate solutions were subsequently combined, then obviously some of the active material separated out and was filtered off.

In repeating the above method of extracting the hormone, but using decidedly more acid, we discovered that the final product was even less soluble in alkali than before. However, the residue taken up in oil proved more potent than a comparable residue prepared by the use of less acid.

Since the use of more concentrated acid gave a more active product, but one less soluble in alkali, it was hoped that solubility could be enhanced by first boiling the chloroform residue with alkali, and then acidifying, steam distilling, etc. The results were interesting, but did not solve the problem of solubility in alkali.

At this stage we came across the papers of Butenandt [1929] who holds that the female hormone occurs in two forms, one having an open ring formation whilst the other is a lactone. To obtain the hormone completely alkali-soluble it is necessary to heat with alkali, so as to convert any lactone modification into the open ring form. On the other hand, heating with acid converts most of the substance into the lactone. Butenandt also states that if the material heated with alkali is extracted with ether, various impurities are removed, but the active material stays behind.

Having formed the opinion that the male and female hormones behave similarly towards chemical reagents, we now hoped that we could purify the

[1] Earlier papers have appeared in the *Proc. Soc. Exp. Biol. Med.* (1929), **26**, 325 and 569, in the report of the *Thirteenth International Physiological Congress* (1929), p. 89, and in the *Amer. J. Physiol.* (1930), **92**, 440.

male hormone by applying Butenandt's procedure with the female hormone. However, it was soon discovered that when the material obtained from male urine was heated with alkali and extracted with ether, most of the activity passed into the ether layer. Here for the first time one could observe a very sharp difference in chemical behaviour between the male and female hormones.

These results also suggested that possibly alkaline extraction of urine had advantages over acid extraction, particularly so in view of the work of Dingemanse [1930]. Actual experiments did not prove this to be the case. On the contrary, considerable loss of activity resulted when the urine was not acidified prior to extraction. It is probable then that the male hormone is present in the urine in two forms: one in the free state, and the other so combined or in such form as to be insoluble in chloroform, and only rendered soluble in this solvent by treatment (hydrolysis?) with acid.

It is probable that alkaline extraction of urine may be used to separate the male and female hormones[1], as Dingemanse points out; but our experiments show that the separation is only complete in so far as the female hormone is concerned, although by the alkali-ether treatment, outlined above, a better yield of the male hormone is obtained.

Out of a mass of experiments performed in connection with this work, we select one of the most recent, since it illustrates a number of points raised in this note.

In this experiment we started with 24 litres of mixed urine, obtained from men under 40 years of age. 6 litres of this urine were directly extracted with chloroform after the urine had become distinctly alkaline, the chloroform was evaporated, and the residue taken up with peanut oil (*a*). The remaining 18 litres were first acidified to Congo red, then strongly acidified (40 cc. conc. HCl per litre of urine), and extracted with chloroform under reflux. One-third of the chloroform extract (corresponding to 6 litres of urine) was evaporated, and the residue taken up with oil (*b*); two-thirds of the chloroform extract (corresponding to 12 litres of urine) was evaporated, heated with 10 g. NaOH in 50 cc. water for two hours, extracted repeatedly with ether, the ether extract divided into two equal portions, the ether in each case evaporated, and the one residue taken up with oil (*c*) and the other with diethylin (diethyl ether of glycerol) (*d*). In each case capons were injected with the equivalent of 300 cc. of urine per day. For each experiment four capons were used. The results were as follows:

(*a*) Percentage increase in comb growth in 5 days				...	12·6
(*b*)	,,	,,	,,	...	26·4
(*c*)	,,	,,	,,	...	19·5
(*d*)	,,	,,	,,	...	19·3

[1] The occurrence of male hormone in female urine and female hormone in male urine can probably be best explained by the occurrence of these hormones in foods. This view is supported by the fact that urine of even very old men still contains small, but appreciable quantities of the male hormone.

Roughly speaking, then, twice as much of the male hormone can be obtained from a strongly acidified urine as from alkaline urine.

Our results also lead to the view that ether extracts much of the male hormone from strongly alkaline solutions (obtained by extracting strongly acidified urine). According to Butenandt this is not true of the female hormone.

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CLXXXVI. PURINOLYTIC ENZYMES OF THE HUMAN ORGANISM.

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In spite of the importance to human pathology of elucidating whether or not the human organism is capable of decomposing uric acid no actually decisive experiments on this question have been described, and the papers published give conflicting results. Pfeiffer [1906] stated that the kidney tissue of adults has a strong uricolytic action. Schittenhelm and Schmidt [1907] found that all tissues of new-born children are able energetically to destroy uric acid, and Cannata [1910] found that the human placenta possesses similar properties. Schittenhelm expressed the opinion that the reason why uricolysis did not normally take place in the human organism was that certain inhibiting substances were present in the body fluids. Folin, Berglund and Derick [1924] came to the conclusion that uric acid can, in normal persons, be oxidised by circulating blood, but that this property is immediately lost by removal of blood from circulation. Folin's experiments indicated, further, that the blood of gouty subjects was not able to destroy uric acid, and on this observation Folin based a theory of the etiology of gout. Flatow [1926] found that shed blood contained a uricolytic substance termed by him urico-oxidase; this however appears to be haemoglobin entering solution on haemolysis of erythrocytes, as is indicated by Gomolinska's work [1928]. Rowinska (unpublished work) showed further that unhaemolysed blood does not, under aseptic conditions, destroy uric acid. Wiechowski [1909], who examined various human organ preparations, was not able to find any uricolytic action, and similar results were obtained by Künzel and Schittenhelm [1908] and by Wells and Corper [1909]. Beck and Truszkowski [1930], using glycerol-chloroform extracts of foetal tissues taken from the 8th to the 40th week of pregnancy, found no evidence of the presence of uricase in unshaken systems.

The above somewhat chaotic results must be ascribed in some cases (Wells and Corper) to a misconception as to the nature of uricase, which was previously thought to be a soluble enzyme, and which was accordingly, in certain of the above researches, sought for in the extracts of organs instead of in the residue after extraction, which according to Truszkowski's work [1930] alone contains this biocatalyst. A second source of error lies in the action of micro-organisms on uric acid in the presence of such weak antiseptics as thymol, sodium fluoride, etc.; to this we would ascribe the results of Pfeiffer, Schitten-

helm and Schmidt, and Cannata. Finally, the disturbing effect of xanthine oxidase was not allowed for in the above experiments on isolated tissues and their extracts. Thus, even if the tissues examined had possessed a feeble uricolytic action, the diminution in the uric acid content of the systems might readily be more than compensated for by the formation of fresh uric acid from purine bodies present in the organ or its extract; *i.e.* uricase cannot with certainty be stated to be absent unless xanthine oxidase is either removed or proved to be absent. As to metabolism experiments, such as those of Folin, in which it was attempted to obtain a balance between injected and excreted uric acid, these cannot be considered to be of decisive value, in view of the fact that uric acid is excreted not only with the urine, but also with the bile, and possibly also directly into the intestines, in which bacterial decomposition may, and in all probability does supervene.

The above critical survey of previous work done in this field shows that from none of the papers hitherto published can it be definitely stated whether or not human tissues are possessed of uricolytic properties either in intra- or extra-uterine life.

As to xanthine oxidase, Wells and Corper [1909] were unable to demonstrate its presence in the human foetus before the 6th month of pregnancy. Beck and Truszkowski [1930] found this enzyme in glycerol-chloroform extracts of foetal tissues from the 8th week of pregnancy up to the full term; the results of these authors are however only qualitative.

For the above reasons it was considered desirable to re-investigate the whole question, applying the methods of preparation of uricase described by the present author [1930]. The establishment of the insolubility of uricase affords a simple method for its separation from soluble xanthine oxidase, and therefore of the examination of the action of these two enzymes separately, so that the results obtained appear to be free methodically from the objections raised to those of earlier workers. The organs examined were the liver and, in some cases, the kidneys. These were taken for the greater part from embryos, and in some cases from healthy adults who had died suddenly from accidental causes. These organs were selected because in mammals other than man and the anthropoid apes either the liver or kidney or both of these organs possess the most pronounced uricolytic activity.

Experimental.

The liver and kidneys were removed as soon as possible after death (0·5–3 hours) and were minced, or, if too small, as in the case of foetuses born before the 5th month of pregnancy, cut up into small pieces, and ground in a mortar with chloroform. Ten times its weight (in the case of the youngest foetus 15–20 times) of water was added to the comminuted tissue, which was left with excess of chloroform during 48 hours, after which part of the suspension was centrifuged, and the uricolytic actions of the whole suspension, of the centrifugate, and of the residue were examined. In view of Schittenhelm's

theory of the presence of substances inhibiting uricolysis, the effect of adding centrifugate to systems containing bovine kidney uricase was also examined (Table I). Uric acid solutions were in all cases prepared by dissolution of uric acid in boiling saturated lithium carbonate. Uric acid was determined colorimetrically by the method of Folin and Denis [1913].

A number of 100 cc. flasks each containing 10 cc. of about 0·2 % lithium urate solution (p_H 8·5) were prepared. To certain of these flasks were added 10 cc. of water and 10 cc. of centrifugate, of a suspension of the residue after centrifuging, of the whole suspension, or of a 2 % suspension of bovine kidney uricase. In addition to these systems, flasks were prepared containing 10 cc. of lithium urate solution and 20 cc. of water, 10 cc. of centrifugate and 20 cc. of water, and finally, 10 cc. of lithium urate solution, 10 cc. of centrifugate and 10 cc. of a suspension of bovine uricase. To each flask 1 cc. of toluene was added, in order to inhibit bacterial growth. The flasks were then tightly stoppered, using rubber corks, and were placed in an Ostwald water thermostat maintained at 37° ± 0·2°, with constant shaking. It has been shown by the author that uricase is most active under these conditions, 1 g. of bovine kidney powder being capable of catalysing the oxidation of 220 mg. of uric acid in 1 hour.

Three livers, taken from a foetus born at term (*placenta praevia*), and from two adult females, aged 27 and 57 years, were examined. Death took place in both cases suddenly, without any previous history of disease, so that the results can be regarded as being typical of normal healthy organs. Systems containing preparations of the foetal liver were shaken for 14·5 hours, whilst those of adult liver were shaken for 24 hours; those systems containing bovine uricase were shaken respectively for 1, 2 and 2 hours. The results given in Table I show that in no case is any diminution in uric acid content demonstrable, except in the presence of bovine uricase. It follows that the human liver is not possessed of even the feeblest uricolytic power. The uric acid content of systems containing centrifugate or whole extract is in all cases slightly higher than that of the control systems or of those to which the residue was added—this difference is due to preformed uric acid present in the extract, as is shown by the results found for the diluted centrifugate alone. The centrifugate has a slight inhibitory influence on the action of bovine uricase; this however, as was shown by the author in a previous paper [1930], may be ascribed largely to increase in viscosity of the medium occasioned by soluble proteins. Since however the corresponding centrifugate of bovine kidney extracts has a considerably greater inhibiting action, Schittenhelm's hypothesis that human organs would be uricolytically active were it not for the presence of inhibitory substances must be abandoned in its entirety.

It appears from the above experiments that during extra-uterine life the human organism does not possess catalysts capable of decomposing uric acid. The possibility exists that such factors may exist prior to birth, at some stage of embryonic development corresponding to that of mammals other than man,

Table I. *Action of preparations of human liver on uric acid.*

No. of exp.	Material	Systems contain: Uric acid cc.	Suspension of residue cc.	Centrifugate cc.	Whole extract cc.	2 % suspension of bovine uricase cc.	Water cc.	Mg. uric acid: Found	Oxidised	Remarks
1	10-month old foetus.	10	10	—	—	—	10	22·8	0·0	
	Systems shaken	10	—	10	—	—	10	24·3 (−1·5)	0·0	
	14·5 hrs. at 40°	10	—	—	10	—	10	24·3 (−1·5)	0·0	
		—	—	10	—	—	20	1·5	—	
		10	—	10	—	10	—	17·55 (−1·5)	6·75	Shaken
		10	—	—	—	10	10	14·7	8·1	1 hr.
		10	—	—	—	—	20	22·8	—	
2	Adult, ♀, aged 27.	10	10	—	—	—	10	18·6	0·0	
	Systems shaken	10	—	10	—	—	10	21·6 (−3·0)	0·0	
	24 hrs. at 37°	10	—	—	10	—	10	21·6 (−3·0)	0·0	
		—	—	10	—	—	20	3·0	—	
		10	—	10	—	10	—	6·35 (−3·0)	15·25	Shaken
		10	—	—	—	10	10	2·43	16·17	2 hrs.
		10	—	—	—	—	20	18·6	—	
3	Adult, ♀, aged 57.	10	10	—	—	—	10	18·6	0·0	
	Systems shaken	10	—	10	—	—	10	20·1 (−0·5)	0·0	
	24 hrs. at 38°	10	—	—	10	—	10	20·1 (−0·5)	0·0	
		—	—	10	—	—	20	0·5	—	
		10	—	10	—	10	—	4·2 (−0·5)	14·8	Shaken
		10	—	—	—	10	10	2·43	16·17	2 hrs.
		10	—	—	—	—	20	18·6	—	

Note. Figures in brackets refer to preformed uric acid.

which might be presumed to possess uricase. Morphologically, such a stage occurs during the early phases of development, before the 8th week of pregnancy; it is in practice not possible to obtain intact embryos from these early stages. Since however Jones and Austrian [1907], and Mendel and Mitchell [1907] found that the appearance of a different enzyme, xanthine oxidase, does not take place till a comparatively late stage of development in pig embryos, it is possible that the disappearance of uricase might be similarly retarded in the human embryo. Przyłęcki and Rogalski [1927–8] reported the appearance of uricase in embryonic chicks on the 2nd day of development and its disappearance on the 10th day.

As to xanthine oxidase, Wells and Corper [1909] came to the conclusion that this does not appear before the 6th month of pregnancy. Beck and Truszkowski [1930] on the other hand demonstrated its presence in all embryos examined, from the 8th to the 40th week of pregnancy; no attempt was however made in this research to obtain other than qualitative evidence of its presence.

The livers and kidneys of six embryos from the 12th to the 40th week of pregnancy were examined for uricase by the method described above, *i.e.* the residue obtained by centrifuging chloroform-water extracts of the finely divided organs was shaken for 24 hours at 37° with lithium urate solution. In no case was any oxidation of uric acid found to take place, so that the conclusions drawn for post-natal organs can be extended to all foetuses after the 12th week of pregnancy.

Table II. *Xanthine oxidase in human liver extracts.*

Week of pregnancy	Mg. uric acid found after leaving 20 cc. of centrifugate 48 hrs. at 25° with 10 mg. of xanthine
12	0·3
18	3·06
20	2·00
24	3·3
28	2·81
40	9·5

The centrifugates were diluted to a volume of about 22 cc., and two 10 cc. portions of each centrifugate were placed in a number of flasks. One flask of each pair was heated at 100° for 3 min. in order to destroy the enzyme. 10 cc. of a 1 % suspension of xanthine and 2 cc. of toluene were then added to each flask, and the flasks, after plugging with cotton-wool, were left for 48 hours at 25°, after which time uric acid was determined. No uric acid was found in those flasks which had previously been heated at 100°, or in those containing kidney extract centrifugates. Varying amounts were found in the systems containing liver extract centrifugates (see Table II). The activity of the respective centrifugates obviously depends not only on the enzyme content of the organ, but also on its dilution, which was necessarily greater for material taken from early pregnancy. It will be seen that little variation exists between the 18th and the 28th week of pregnancy, whilst for the 40th week practically all the xanthine added (10 mg.) had been converted into uric acid.

Summary.

1. The livers of a new-born child and of two adults have been examined for uricase, which is shown to be absent.

2. The livers and kidneys of six foetuses from the 12th to the 40th week of pregnancy have been similarly examined; uricase is in all cases absent.

3. Xanthine oxidase is absent from the extracts of the kidneys of the above foetuses.

4. Xanthine oxidase has been found in all liver extracts from the 12th to the 40th week of pregnancy; Wells and Corper's view that this enzyme does not appear before the 6th month of pregnancy is therefore incorrect.

The author wishes to express his gratitude to Prof. St J. Przyłęçki for his interest in this research and to Docent H. Beck for supplying the material.

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CLXXXVII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. IX.

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This communication deals in the main with two aspects of the antiscorbutic factor, namely, its fractionation and the mechanism controlling its spontaneous inactivation.

The method of fractionation introduced some years ago by the author was based on the precipitation of the active principle by lead acetate at p_H 7–7·2 after removing the acids from lemon juice and clearing the resulting solution with alcohol or neutral lead acetate or with both in succession. In the early stages [1924] basic lead acetate was employed as the precipitating agent, but as the work progressed it became apparent that very unequal results were obtained, which were then ascribed to the alkalinity of the basic lead acetate, and consequently a modification, which consisted in the precipitation of the vitamin at p_H 7–7·2, was introduced [1927]. Further experiments have, however, shown that the slightly alkaline reaction of the medium was not alone responsible for the low activity of some of the fractions obtained [1928, 1929] and that some uncontrolled factor, possibly varying with the character of the juice employed, was involved. It was therefore felt that evidence sought along this path might not only yield information conducive to the preparation of fractions of more uniform activity, but might afford knowledge with regard to the general complexity of the problem. Results obtained so far seem to favour the view that the variable activity obtained by precipitation with lead acetate in the neutral zone is controlled by the character of the juice. Thus a quantity of lead acetate which precipitates the greater part of the active principle in one juice fails to bring down much of the vitamin in another case, but, by adjusting the quantity of the precipitating agent, the activity of the precipitate obtained from the juice can be greatly enhanced.

What the factor in the lemon juice which controls the precipitation of the antiscorbutic factor is, has not so far been ascertained, but the following results will make it plain that the fractionation of the principle is a complicated subject requiring critical judgment in its manipulation and in the interpretation of the results.

Since the publication of the above method of fractionation, experiments

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dealing with its extension and its application to other sources of the anti-scorbutic factor have been published by several workers. Their results are, however, quite contrary not only to what the author's experience would lead him to expect, but to his actual observations; which suggests that it is necessary to exercise caution in order to interpret correctly the available evidence on the subject. To emphasise this necessity, the experiments on the fractionation of the vitamin are here communicated.

The experiments described in the section of this communication dealing with the spontaneous inactivation of the antiscorbutic factor are an extension of the work recorded recently [1929]. In that communication it was shown that the addition of autoclaved decitrated lemon juice or of quinol to unheated decitrated juice accelerated the destruction of both the reducing principle[1] and the antiscorbutic factor of the mixture at neutral reaction. It was further found that the iodine-reducing capacity of the autoclaved solution increased[2] and that the activity of the peroxidase present in lemon juice persisted after the heating. A plausible explanation of the accelerated inactivation of the vitamin after autoclaving presented itself. A substance, probably of a phenolic character, was formed in the autoclaving which on oxidation in the air acquired the property of acting in conjunction with the thermostable peroxidase as an oxidiser of the reducing substance, the disappearance of which, as has been shown [1927, 1928], precedes the loss of the antiscorbutic activity. The experiments described in this communication support this view. The addition of benzoquinone, quinhydrone or ethereal extracts from autoclaved decitrated juice accelerated the inactivation of the vitamin in unheated decitrated juice to much the same extent as if the juice itself had been autoclaved.

Williams and Corran [1930] have recently shown that in the case of lemon juices adjusted with hydrochloric acid to various hydrogen ion concentrations ranging from p_H 0·6 to 2·2 and stored for 7–8 months, the antiscorbutic activity greatly deteriorated when the p_H was less than 1·8. Experiments are described here to show that there is no appreciable destruction of the reducing principle or of the antiscorbutic factor in decitrated lemon juice kept at p_H 1 or less for one week, so that the destruction of the vitamin observed by these workers, if it be directly due to the acidity and not to any other factor inherent in the conditions under which they were working, must be comparatively slow.

[1] Szent-Györgyi [1928] suggests that the reducing principle is probably identical with the hexuronic acid which was isolated by him from the adrenal cortex and from fruit and vegetable juices. No direct evidence has yet been produced to substantiate this suggestion. Until such evidence is available the author proposes to use the expression "reducing principle."

[2] Experiments performed since the publication of this observation have shown that there is not so much an increase in the decolorising capacity as increase in the speed at which the decoloration of the iodine takes place. There is no very significant difference between the amounts of iodine that are decolorised by autoclaved and unheated juice if an excess of the reagent is added and the residual iodine is titrated back after 24–48 hours. It must, however, be noted that some destruction of the reducing principle, which also decolorises iodine, takes place in the autoclaving.

Experimental.

The variable activity of antiscorbutic fractions from lemon juice.

In the concentration and purification of the antiscorbutic factor the author has always adopted the procedure of assessing the activity of a preparation by reference to its equivalent of the original lemon juice. In the early experiments it was found that doses equivalent to 1·5 cc. of the juice were capable of keeping young guinea-pigs alive for periods of about 60 days; these animals were, however, not always free from scurvy at that time. Although the same dose of lemon juice or of decitrated juice is capable of preventing the development of any macroscopic scorbutic lesions and even inducing good growth during this period, it is assumed, considering the risk of loss in activity involved in the preparation, that the best part of the vitamin of the juice in such cases is obtained in the final fraction. As already stated, experience has shown that preparations of such high activity could not always be obtained in spite of the fact that, as far as it was possible to judge, uniform conditions were observed in the manipulation. It would require too much space to describe in detail all the fractionations performed during the last few years, but an analysis of these will afford a fair view of the variations in activity. Before discussing the results it is, however, necessary to give a detailed description of the method of preparation, though mention of it has already been made in previous publications of this series, since it is found that small deviations from the procedure often give negative results in the case of juices capable of yielding active fractions. The details given were applied to small batches of juice varying from 50 to 200 cc. These were always prepared and dosed daily except during the week-end. Less frequent preparation and storage of large batches led mostly to considerable loss in activity.

The acids are removed from the lemon juice by the addition of an excess of precipitated chalk and the mixture is allowed to stand at room temperature for about 1 hour. The liquid is then filtered through a Büchner funnel and to the filtrate (about p_H 6·4) an equal volume of neutral lead acetate is added. After immediate filtration through a Büchner funnel this filtrate (p_H 5·0–5·4) is brought up to p_H 7·2 by a solution of 6 % ammonia. The precipitate formed is quickly centrifuged and taken up in 10 % acetic acid, yielding usually a clear or slightly opalescent solution. The lead is then removed from the solution by the addition of just sufficient saturated magnesium sulphate (3–5 cc. for 50 cc. of decitrated juice) and subsequently of 2 volumes of 96 % alcohol and allowing the mixture to stand for 10–15 mins. The filtrate after being evaporated under reduced pressure at 40°–50° to a volume of 2–5 cc., when the excess of acetic acid has been distilled off, is diluted with distilled water to a convenient volume. Enough 20–25 % ammonia is then cautiously added to reduce the acidity to p_H 5·0–6·0 and the solution is brought up to its original volume with distilled water. The preparations must be administered to the animals immediately, since the inactivation of purified active solutions proceeds much

more quickly than does that of original juices of similar activity [Zilva, 1928]. The decomposition of the lead precipitate with H_2S instead of magnesium sulphate yields less satisfactory results. The above manipulation should not take more than 3–4 hours.

The 11 tests reviewed here in which over 95 animals are involved cover a period of several years and were performed at various seasons during the years 1925–30. All the animals referred to, unless otherwise stated, were chloroformed after 55–60 days and the degree of scurvy assessed at the *post mortem* examination.

Of the animals receiving a dose equivalent to 1·5 cc. of lemon juice, in six out of eleven sets, comprising 20 animals, hardly any delay in the onset of, or fatal termination due to, scurvy had been noticed. In the remaining five sets, comprising 17 animals, all the guinea-pigs survived the experimental period. In 6 of these animals hardly any scurvy could be detected, the others all showed marked scurvy at the *post mortem* examination.

Of the animals receiving a dose equivalent to 3 cc. in four sets out of nine, comprising 13 guinea-pigs, hardly any delay in the onset of and fatal termination due to scurvy could be observed. The remaining five sets comprising 13 animals, of which only 1 guinea-pig showed marked scurvy at the *post mortem* examination, survived the experimental period.

Of all the animals receiving a dose equivalent to 5 cc., in two sets out of eleven, comprising 6 animals, hardly any delay in the onset of and fatal termination due to scurvy could be observed. The remaining nine sets, comprising 22 animals, of which only 1 guinea-pig showed definite scurvy at the *post mortem* examination, survived the experimental period.

Out of one set of 4 guinea-pigs receiving a dose equivalent to 10 cc. of lemon juice, 1 animal died of an intercurrent disease without showing any signs of scurvy after 64 days. The remaining animals were chloroformed after 92–97 days and were found almost entirely protected from scurvy.

No correlation was found to exist between the activity of the various fractions and the season when they were prepared. As very little time usually elapsed between the arrival of the lemons in this country and their utilisation in the experiment, the date of the test can be used more or less as an indication of the time of their picking. Unfortunately it was difficult to obtain detailed information about cultural conditions owing to the fact that the fruit was bought in the open market. The following experiments, however, throw some light on one of the causes responsible for the low activity of some of the preparations.

The influence of various quantities of neutral lead acetate on the fractionation of the antiscorbutic factor.

It will be noticed that an equal volume of saturated neutral lead acetate was added to the decitrated lemon juice in the preparation of the antiscorbutic fractions in the experiments described above and that in the early experiments preparations were obtained which showed considerable potency in

doses equivalent to 1·5 cc. of the original lemon juice. In the search for the cause of the unequal activity in some of the later preparations, it was observed that when an equal volume of neutral lead acetate was employed a fraction was sometimes obtained possessing very little reducing capacity (1–3 cc. of 0·02 % phenolindophenol per 5 cc. of the solution) and, as anticipated, such fractions were found almost inactive antiscorbutically. On the other hand, it was possible to adjust the quantity of neutral lead acetate so that the reducing capacity of the fraction was increased with a simultaneous increase in antiscorbutic activity. The experiment, which is represented graphically in Fig. 1,

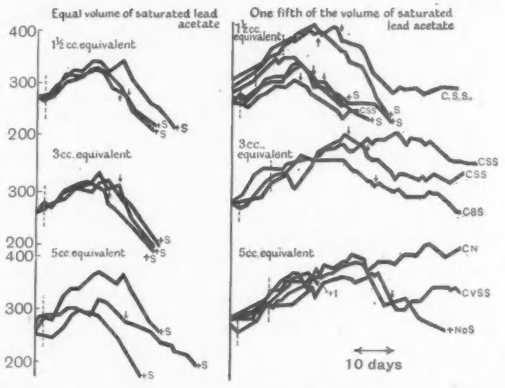

Fig. 1. +, died; *C*, chloroformed; *S*, scurvy; *SS*, slight scurvy; *VSS*, very slight scurvy; *N*, normal; ↓, onset of scorbutic symptoms; ⟊, beginning of the daily administration of the dose.

demonstrates this point. It is seen that when an equal volume of neutral lead acetate was added, the juice did not show any activity even in doses equivalent to 5 cc. of the original juice, a quantity which decolorised on the average only 2·5 cc. of 0·02 % phenolindophenol. On the other hand, when the amount of neutral lead acetate was reduced to one-fifth of the volume of the juice, all other conditions remaining the same, the antiscorbutic potency was greatly enhanced. An equivalent of 5 cc. of this preparation reduced on the average about 7·5 cc. of 0·02 % phenolindophenol. The reducing capacity for phenolindophenol of the final fraction may, therefore, be utilised in this case as an index for the determination of the optimum amount of neutral lead acetate necessary to employ in the manipulation in order to achieve good antiscorbutic activity. So far it has been found that amounts of reagent varying from 18 % to 25 % of the volume of the juice are required to achieve the best results, although, as is evident from the earlier experiments, good potency can sometimes be obtained with equal volumes of lead acetate. When a uniform supply of lemons is used, the same amount of precipitating reagent can be used during the 2 months' duration of the test; the reducing capacity of the

fraction as shown by its reduction of phenolindophenol varies in such cases within certain limits, but is always above the minimum which experience has shown is necessary to ensure potent fractions, namely, above about 6 cc. of 0·02 % of the indicator for an equivalent of 5 cc. of juice. When the batch of lemons is not uniform in character, as sometimes happens, the quantity of neutral lead acetate used must be adjusted according to the daily variation in the titrations of the reducing capacity of the fractions.

It must here be emphasised that an increase in the reducing capacity in preparations obtained as described above, as measured by the decoloration of phenolindophenol, above the minimum necessary to insure antiscorbutic activity, in no way indicates a proportionate increase in the antiscorbutic activity. Beyond this limit this reducing capacity is more an index of the stability of the antiscorbutic potency.

Attempts at further purification of the active factor.

The first obvious procedure to attempt in order to effect further purification was the reprecipitation of the active fraction, but this had to be given up since no activity could be obtained in the second precipitate. Two experiments are cited here to illustrate the difficulties.

In the first experiment after taking up the active precipitate in 10 % acetic acid, 25 % ammonia was added cautiously until the neutral zone was almost reached and then the neutralisation was completed with 6 % ammonia. No precipitate was formed at p_H 7·0, as it was in the case of the first precipitation. When the p_H was raised to about 8 a precipitate began to form, the maximum precipitation being obtained between p_H 8 and 9. This precipitate was decomposed and tested in the usual way and, as will be seen from Fig. 2, it

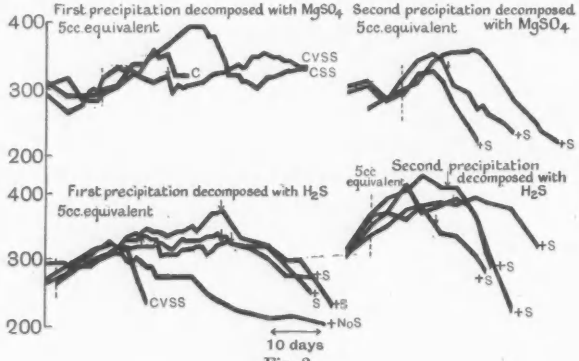

Fig. 2.

was found to be inactive in a dose equivalent to 5 cc. of the original juice, whilst the first precipitate from the same juice was active in this dose. An equivalent of 5 cc. of the first precipitate reduced 7·5 cc. of 0·02 % phenolindophenol. A similar quantity of the second precipitation reduced 2·5 cc.

In the second experiment the first lead precipitate was decomposed with H_2S, and the H_2S removed by concentration of the resulting solution at 40°–50° under reduced pressure to a small volume. After diluting to its original volume, it was brought up to p_H 7–7·2 with 6 % ammonia. In this case the maximum precipitation was obtained at this reaction. The precipitate was decomposed in the usual way and, as will be seen from Fig. 2, there was a marked diminution in the activity in this second precipitate. The activity of the first precipitate in this experiment was not very high but this is not unusual when the lead precipitate is decomposed with H_2S instead of magnesium sulphate.

The above experiments, characteristic of several performed on these lines, are quoted here in view of the fact that Grettie and King [1929] and Sipple and King [1930] claim to have obtained very little loss in activity in the second precipitation, although the fractions were not even prepared every day but at least once a week and stored under anaerobic conditions before being tested.

Bezssonoff also claims to have obtained an active fraction from cabbage juice after several precipitations. This writer [1922, 1923] asserted that by clearing cabbage juice with neutral lead acetate, decomposing the precipitate with H_2S and concentrating to dryness under reduced pressure and low temperature, an active powder was obtained. Later [1925], he applied the author's principle but, instead of using basic lead acetate, precipitated the vitamin from the juice previously cleared with lead acetate and acetic acid by adjusting the reaction of the solution with sodium hydroxide to p_H 8·2. Further purification was carried out by taking up the precipitate in acetic acid, precipitating it twice more, decomposing it with H_2S and extracting with alcohol and with acetone. This procedure it is claimed yielded active crystals.

Considering the disappointment experienced by the author in attempting to obtain any antiscorbutic activity in the second precipitate, and the difficulty of manipulating active fractions in general without impairing their activity, the results obtained by Bezssonoff seemed rather surprising. This feeling was further strengthened by many observations that cabbage juice was the least stable source of the antiscorbutic factor so far handled. The experiments described below show that, in fact, in the author's hands, extremely disappointing results were obtained in the fractionation of this juice.

The outer green leaves without the stalk of the cabbage were thoroughly minced and pressed out through chain cloth by means of a hand press. The juice was then centrifuged to remove suspended matter and the untreated supernatant liquid (preparation A) was tested as a control. To another portion of this centrifuged juice 35 % of its volume of a saturated solution of neutral lead acetate was added, a quantity of reagent with which was usually obtained the highest reducing capacity in the resulting supernatant solution with this material, and the heavy precipitate centrifuged and rejected. The supernatant liquid was then decomposed with H_2S and the filtrate concentrated to a small volume under reduced pressure at a temperature of 40–50° in order to remove the H_2S. After diluting the residue to its original volume with distilled water

Page 1693, line 17:
for precipitate *read* filtrate

and filtering off the little suspended matter, this preparation (preparation B) was ready for dosing. To another portion of the supernatant fluid, from the neutral lead precipitate, which was not decomposed with H_2S, normal sodium hydroxide was added until the reaction was brought up to p_H 8·2. The precipitate formed was suspended in water, decomposed with H_2S, concentrated as before to a few cc. and made up to its original volume with distilled water (preparation C) and dosed. To a portion of this solution 35 % of its volume of neutral lead acetate was added, and the reaction was again adjusted with sodium hydroxide to p_H 8·2. The precipitate thus formed was decomposed and treated as before and the resulting preparation (preparation E) tested. These preparations were made up daily, except during the week-end when double doses were given, and were administered to guinea-pigs as soon as they were ready. Doses equivalent to 1·5 cc., 3 cc., 5 cc. and 7 cc. were tested in each case. Preparation A was fully protective for 58 days even in equivalents of 1·5 cc. and promoted growth. Preparation B was protective in equivalents of 7 cc. doses only, very slight protection was afforded by the equivalent dose of 5 cc. of juice; in preparation C only one animal receiving an equivalent dose of 7 cc. lingered on for 55 days and finally died of scurvy, all the other animals in this test succumbed to the disease in 30–40 days. Preparation E showed no signs of activity even in doses equivalent to 7 cc. of the juice.

It is thus seen that although small batches were worked up daily under conditions which made it possible to take the greatest precautions against the spontaneous inactivation of the vitamin, no significant activity could be demonstrated even in the first precipitate. This result was not unexpected since this cabbage juice was found to possess, after the removal of the chloroplasts and proteins, a reducing capacity which varied somewhat with the amount of juice yielded by the leaves often from day to day within very wide limits (4–23 cc. of phenolindophenol per 5 cc. of solution) and which was usually low as compared with lemon juice and swede juice.

Even batches of cabbage juice which showed the highest reducing titres after being cleared with neutral lead acetate very rarely yielded a second precipitate with a reducing capacity characteristic of active fractions. It is conceivable that higher activity could have been obtained in preparation B and possibly some activity in the first precipitate (preparation C) if a juice of uniform high reducing capacity were obtained after clearing with neutral lead acetate. The above experiments, however, demonstrate the difficulty of preparing active fractions beyond the first precipitation in the neutral or slightly alkaline zone, even in highly reducing juices.

The spontaneous inactivation of the antiscorbutic factor in autoclaved decitrated lemon juice.

The series of experiments about to be described were instituted with the object of testing the hypothesis that the accelerated inactivation of the antiscorbutic factor in neutral solution produced by the previous autoclaving of

the decitrated lemon juice is due to a chain of reactions in which the thermostable peroxidase and a substance formed during the heating are concerned. Previous attempts [Zilva, 1929] have shown that the addition of autoclaved decitrated lemon juice or of quinol to unheated juice had a similar inactivating influence. In these experiments the effects of the addition of quinhydrone, benzoquinone or an ethereal extract from autoclaved juice were studied. In Exp. 1, quinhydrone (30 mg. per 50 cc. of juice) was added and the solution kept at p_H 7 for 24 hours in the cold room before dosing. In Exp. 2, benzoquinone (10 mg. per 50 cc. of juice) was added and the solution stored as in Exp. 1. In Exp. 3 the ethereal extract from decitrated lemon juice which had been heated at 143° for one hour in an autoclave was added to unheated decitrated lemon juice and stored in the cold room for 7 days at p_H 7. The preparation and addition of this extract were carried out as follows.

50 cc. of autoclaved decitrated lemon juice were shaken in an atmosphere of nitrogen with 75 cc. of ether for about 45 min. The separated ethereal layer was then added to 50 cc. of decitrated juice, adjusted to p_H 6, and the ether removed by means of a water-pump at room temperature. The resulting brown solution contained a trace of matter in suspension which was not filtered off. After adjusting the reaction to p_H 7, the preparation was stored at this reaction for 7 days before dosing. The ethereal extract did not reduce phenolindophenol but decolorised iodine.

The addition of the quinhydrone or the benzoquinone had a very deleterious action on the reducing principle, since hardly any phenolindophenol was decolorised after 24 hours' storage at p_H 7. The oxidising effect of the ethereal extract from the autoclaved juice was slower but the reducing principle was usually absent after 7 days' storage.

In Exps. 1 and 2, 1·5 cc., 3 cc. and 5 cc. doses were tested, using three guinea-pigs on each dose. In Exp. 3, the 3 cc. and 5 cc. doses only were tested on the same number of animals. All the guinea-pigs in the above experiments succumbed to scurvy within one month. A control set of animals showed that the decitrated lemon juice was active immediately after the addition of the ethereal extract.

The effect of hydrochloric acid on the stability of the antiscorbutic factor.

Experience has shown that the manipulation of the antiscorbutic factor in a medium of high hydrogen ion concentration has, presumably, no deleterious effect on the vitamin within short periods of time. In view, however, of Williams and Corran's [1930] observation that lemon juice, to which hydrochloric acid was added to lower the p_H to less than 1·8, when stored for a few months lost a great part of its antiscorbutic potency, it was thought necessary to devise an experiment with the direct object of ascertaining whether any appreciable loss takes place under these conditions of acidity in the space of a week.

The reaction of the daily doses of the decitrated lemon juice was adjusted to approximately p_H 1 by the addition of 4*N* hydrochloric acid. The limits during the entire testing periods were p_H 0·96 and p_H 1·12. The solution was then stored in the cold room for 7 days under aerobic conditions. At the end of this time the hydrogen ion concentration was once more determined and it was usually found that when a change in reaction did take place it was of the order of no more than 0·1 p_H. During the storage the reducing capacity of the juice fell from about 25 cc. to about 16 cc. of phenolindophenol per 5 cc. of juice. The biological test (1·5 cc., 3 cc. and 5 cc. with three animals on each dose) revealed that the guinea-pigs were protected from macroscopic lesions of scurvy for 60 days and that all the animals grew during the test period. The loss in the antiscorbutic potency during a week was, therefore, hardly appreciable. The phenolindophenol titration figures seem to suggest further that the loss of the reducing principle was even somewhat smaller under the above conditions of storage than when decitrated lemon juice is stored at p_H 7. An experiment in which the juice was stored for a week at p_H 0·6–0·8 yielded similar results.

Thanks are due to Miss M. F. Bracewell for carrying out the electrometric determinations in this experiment.

Discussion.

The experiments described in this communication indicate to some extent the difficulties associated with the fractionation of the antiscorbutic factor. Whilst these difficulties may impede the further concentration of the active principle they are at the same time instrumental in the unfolding of an aspect of the subject which promises to reveal some very interesting facts concerning the character and mode of action of the vitamin. The results recorded by the author during the last few years leave little doubt that the activity of the vitamin is controlled by the presence of the reducing principle which in itself is not antiscorbutically active. Preliminary attempts to replace the reducing principle after mild oxidation have so far failed to activate antiscorbutic fractions which have lost their potency through the destruction of the former. Although this failure, considering the complexity of the subject, may not be final, it is nevertheless not unreasonable to assume at this stage of the inquiry that the reducing substance acts irreversibly as a protective agent against the oxidation of the vitamin. In addition, further evidence is accumulating which suggests that, at least in a neutral medium, the reducing substance may be oxidised by means of a peroxidase and a substance acting like an organic peroxide, the latter being formed from a compound present in the autoclaved juice by exposure to air. This compound is possibly of a phenolic character and formed in the process of autoclaving, since the addition not only of the ethereal extract of autoclaved juice, but of quinol or its immediate oxidation products, quinhydrone or benzoquinone, accelerates the destruction of the reducing principle and of the vitamin. The chemistry of this and of the reducing principle is being studied further.

If it be assumed that the destruction of the antiscorbutic factor takes place in the series of reactions described above, then the stability of an active solution can be roughly gauged by the amount of phenolindophenol it reduces. Iodine, on the other hand, is decolorised both by the reducing principle and by the substance or substances formed on autoclaving, and consequently the amount reduced does not necessarily indicate the stability of an antiscorbutic fraction; it may under certain circumstances even serve as an index of its impending speedy inactivation. It is, in fact, possible that the relation of the decolorising capacity of iodine to that of phenolindophenol may be found to be indicative of the stability of an antiscorbutic solution even before heating.

So far this hypothesis of the inactivation of the antiscorbutic factor has been discussed here mainly in relation to the concentration of the active principle with the ultimate purpose of isolating and identifying it. If a chain of reactions similar to that outlined above does actually take place in the expressed juice, may it not be, as has already been suggested [Zilva, 1927], an indication that a cycle of similar reactions, in which the vitamin forms a link, takes place in the living cell of the plant? Further, may not the onset of scurvy in the animal be prevented not by the ingestion of a single principle, which the organism cannot synthesise, but by a number of links active in the cycle of reactions of the plant cell? It is hoped that information obtained in the inquiries which have been pursued by the writer and his colleagues on the function of the vitamin in the plant and in the animal may answer this.

Summary.

1. Several years' work on the fractionation of the antiscorbutic factor is reviewed and it is shown that variable activity is obtained under presumably the same conditions of fractionation.

2. Batches of lemon juice sometimes yield fractions of low or no antiscorbutic activity when equal volumes of a saturated neutral lead acetate solution are used in the manipulation. It is, however, possible by adjusting the quantity of the precipitating reagent to obtain from the same juice preparations of high activity. The conditions must therefore be adjusted in each case in order to obtain uniform high activity.

3. Fractions of which the phenolindophenol decolorising capacity falls below a certain limit are found antiscorbutically inactive. This reducing property, however, is not an index of antiscorbutic activity itself but serves rather as an indication that the vitamin has been protected from inactivation.

4. The reprecipitation of an antiscorbutic fraction from lemon juice or from cabbage juice with lead acetate in the neutral or slightly alkaline zone (p_H 8–9) yields an inactive preparation.

5. The addition of an ethereal extract from autoclaved lemon juice, which decolorises iodine but not phenolindophenol, of quinhydrone or of benzoquinone to unheated lemon juice accelerates markedly the destruction of the reducing principle and of the antiscorbutic factor in a neutral medium in the presence of

air. It is possible that a substance of phenolic character, oxidisable in the air, is formed in the process of autoclaving. This compound in its oxidised form destroys, in conjunction with the peroxidase present in the juice, the reducing principle that acts as an agent for the protection of the antiscorbutic factor.

6. Decitrated lemon juice kept aerobically at p_H 1 or at p_H 0·6–0·8 for 7 days does not lose its reducing capacity for phenolindophenol to a greater extent than when kept at p_H 7 and shows little loss of its antiscorbutic activity.

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CLXXXVIII. THE DENATURATION OF PROTEINS.

VII. DENATURATION IN THE PRESENCE OF ALCOHOL.

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Although it has been known for many years that the addition of alcohol to an aqueous solution of a denaturable protein accelerates the rate of denaturation, relatively little quantitative work seems to have been carried out in connection with the process. For this reason a systematic study of denaturation in a number of alcohol-water mixtures should be of value, more particularly as the conditions involve alterations in such factors as hydration and dielectric capacity of much greater magnitude than can be realised in the case of denaturation in aqueous solutions containing dilute acids, bases or salts.

In the present paper the investigation is confined to a study of the effect of alcohol upon heat denaturation, that is on denaturation in the neighbourhood of the neutrality point of water.

The protein used was haemoglobin, prepared from fresh defibrinated ox blood following the method employed by Lewis [1926] and Cubin [1929]. A method similar to that employed by these workers was adopted for determining the increase in denaturation with time. The absolute alcohol employed was digested with activated quicklime (prepared by roasting marble at 800–1000° in an electric oven for some hours), and distilled several times. The p_H of the solutions was determined by means of the quinhydrone electrode following the technique of Corran and Lewis [1924]. In using this method for determining the p_H, the usual "constant" in the E.M.F. expression cannot be employed in the calculations since the solubility of the quinhydrone varies with the composition of the alcohol-water mixture. A different "constant" must therefore be used for each alcohol concentration. In the present work these "constants" were obtained by extrapolating the values found by Millet [1927]. No change in p_H was observed during the course of the denaturation process, which agrees with results obtained for this protein in the absence of alcohol.

The concentration of alcohol was varied from 2 to 30 vols. % and the temperature at which the reaction was investigated was varied from 30° to

60·5°. The method of damping the reaction was by very rapid cooling in an ice-bath. Owing to the possibility of denaturation occurring at room temperature it was necessary to keep the solutions in the ice-bath except for the filtration and estimation processes which were carried out as rapidly as possible.

The results are given in Tables I to V. Satisfactory unimolecular velocity constants were obtained in all cases. Consequently the mean values alone are given. The unit of time employed is the second.

Table I.

Denaturation in 2 % alcohol

	p_H	$k \times 10^4$
(*a*) 60·5°	6·00	5·06
	6·33	3·23
	6·59	2·46
	6·76	2·28
	7·11	3·30
	7·25	3·79
(*b*) 55°	5·49	2·03
	6·01	0·60
	6·46	0·31
	6·62	0·28
	7·33	0·72
	7·41	0·84

Table II.

Denaturation in 4 % alcohol

	p_H	$k \times 10^4$
(*a*) 60·5°	5·67	7·16
	5·92	6·72
	6·07	5·09
	6·36	4·44
	6·48	4·81
	6·76	6·41
	7·08	8·22
(*b*) 55°	5·43	3·12
	5·99	0·84
	6·17	0·63
	6·28	0·51
	6·58	0·62
	7·06	1·45
	7·74	2·78

Table III.

Denaturation in 10 % alcohol at 55°

p_H	$k \times 10^4$
5·36	10·6
5·78	5·32
6·04	3·46
6·21	3·26
6·35	3·82
6·52	4·24
7·00	6·40

Table IV.

Denaturation in 20 % alcohol

	p_H	$k \times 10^4$
(*a*) 45°	5·19	18·6
	5·50	7·88
	6·01	4·56
	6·57	4·52
	6·78	5·44
	7·18	9·99
(*b*) 40°	5·54	0·52
	5·88	0·33
	6·09	0·27
	6·27	0·27
	6·58	0·35
	6·71	0·43
	7·08	0·72

Table V.

Denaturation in 30 % alcoho

	p_H	$k \times 10^4$
(*a*) 33°	5·82	13·2
	5·90	8·40
	6·04	4·02
	6·10	4·26
	6·32	5·24
	6·43	6·28
	6·75	8·68
(*b*) 30°	5·74	1·98
	6·04	0·60
	6·14	0·67
	6·36	0·84
	6·50	0·95

The general effect produced by alcohol is illustrated in Fig. 1 which for the sake of clearness gives only a selection from the data recorded, namely data from Tables I, II, III and V.

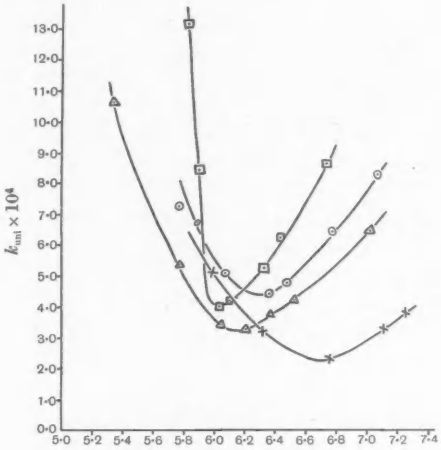

Fig. 1.

The denaturation of haemoglobin in the presence of alcohol.
—×—×— represents denaturation in 2 % alcohol at 60·5°.
—⊙—⊙— „ „ 4 % „ 60·5°.
—△—△— „ „ 10 % „ 55°.
—⊡—⊡— „ „ 30 % „ 33°.

From the above results three facts of importance emerge. In the first place the speed of denaturation is increased by the presence of small amounts of alcohol. This is seen from Table VI, in which the values of the velocity constant in the presence of alcohol are compared with those in the absence of alcohol. The values quoted in the presence of alcohol are taken from the curves obtained by plotting the observed results. The results in the absence of alcohol at 60·5° were obtained by Lewis [1926]. Those given for the temperatures lower than 60·5 and in the absence of alcohol have been calculated on the

Table VI. *The variation in velocity constant with alcohol content.*

	$k \times 10^4$ at 60·5°			$k \times 10^4$ at 55°			
p_H	Water	2 % alc.	4 % alc.	Water	2 % alc.	4 % alc.	10 % alc.
6·0	3·55	5·06	5·70	0·46	0·60	0·84	3·75
6·5	1·53	2·69	4·88	0·20	0·30	0·52	4·24
7·0	1·03	2·85	7·72	0·14	0·37	1·30	6·40

	$k \times 10^6$ at 45°		$k \times 10^7$ at 40°		$k \times 10^6$ at 33°		$k \times 10^7$ at 30°	
p_H	Water	20 % alc.	Water	20 % alc.	Water	30 % alc.	Water	30 % alc.
6·0	0·97	456·0	1·27	291·0	0·0066	404·0	0·0179	600·0
6·5	0·42	420·0	0·44	322·0	0·0029	655·0	0·0077	980·0
7·0	0·28	720·0	0·27	643·0	—	—	—	—

basis that the critical increment between the p_H values of 6·0 and 7·0 is 80,000 cals. for haemoglobin.

The second fact which emerges from Tables I to V is that the presence of alcohol causes the minimum point in the p_H-velocity curve to shift from the neutrality point of water (p_H 6·99) to the acid side. This shift is considerable with small amounts of alcohol and increases slowly with further addition of alcohol. This is shown in Table VII.

Table VII. *Shift of the minimum point with alcohol content.*

Alcohol content in cc. per 100 cc. total volume	p_H of minimum point
0	6·99
2	6·70
4	6·40
10	6·20
20	6·16
30	6·01

The third fact which emerges from the results obtained is that the critical increment of the denaturation process increases with increasing alcohol concentration. This is shown by Table VIII. The values of the critical increment were calculated in the usual way, and hold for the whole of the p_H range investigated.

Table VIII. *Variation in critical increment with alcohol content.*

Alcohol content in cc. per 100 cc. total volume	Critical increment (cals.)
0	80,000
2	83,000
4	85,000
20	105,000
30	117,000

The increase in critical increment in 2 and 4 % alcohol solutions is so small that it might quite easily be attributed to the experimental error, but the increase in the case of 20 and 30 % alcohol solutions is far too large to be explained in this way.

Discussion.

It will be noted that the critical increment (obtained in the ordinary way) increases with alcohol content whilst at the same time the velocity of denaturation likewise increases. This is contrary to what would be expected on the basis of statistical theory as applied to chemical reactions. The origin of the discrepancy must lie in the fact that the influence of temperature is not wholly operative in the ordinary sense, namely, in increasing the probability of rupture of a certain number of bonds which remain essentially of the same type and character over a range of temperature. Thus an increase in temperature may not only increase the probability of rupture, in the usual statistical sense, of the chemical bonds involved in denaturation but likewise may

either release, *i.e.* render available, a number of such bonds or cause a diminution in cohesion on the part of the colloidal unit as a whole. In other words the influence of temperature may be at least twofold. If this be the case the observed critical increment would be a composite quantity and could not be treated without further analysis in the usual statistical manner. It is hoped to examine this point in a subsequent publication from this Laboratory.

As regards the variation of the critical increment with alcohol content the following suggestion may be made. The dielectric constant of water is 81 at 20°, that of pure alcohol is 26, and that of an alcohol-water mixture lies between these two values. One result of the fall in dielectric constant on adding alcohol to the medium will be an increased difficulty in breaking down a bond, particularly if it be dipolar in nature. It seems possible that the increased critical increment may be due to this cause. If E_w is the critical increment in water and E the observed critical increment in a given alcohol-water mixture whose dielectric constant is D, then if D_w is the dielectric constant of water, we should expect on the simplest grounds that

$$E = E_w \cdot \frac{D_w}{D},$$

or

$$ED = E_w D_w = \text{constant}.$$

By interpolation of the data given in the International Critical Tables, Vol. 6, p. 98, the values of the dielectric constant at 20° for varying alcohol concentration are obtained. Using the equation

$$D_t = D_\theta [1 - \alpha (t - \theta)],$$

where D_t and D_θ are the values of the dielectric constant at the temperatures t and θ respectively, and α is a coefficient dependent only on the medium, the value of α for pure alcohol was calculated from the results of Rimbach and Weitzel [1912]. Since the value of α so obtained was found to be near to that for water, it was assumed that the values for alcohol-water mixtures were situated linearly between these two values, and these were calculated accordingly. Employing the values of α so obtained the dielectric constant was corrected to the temperature at which the critical increment was measured, in the case of each alcohol concentration. The values of $E \times D$ were then calculated and are given in Table IX, in the column headed $E \times D_{\text{corr}}$.

Table IX.

Alcoho volume %	E cals.	$D_{20°}$	$E \times D_{20°} \times 10^{-6}$	D corrected for temp.	$E \times D_{\text{corr}} \times 10^{-6}$	Wt % water (C_w)	$E \times D \times C_w \times 10^{-8}$
0	80,000	81·0	6·48	66·2	5·30	100·0	5·297
2	83,000	79·5	6·60	64·9	5·39	98·42	5·302
4	85,000	78·2	6·57	63·7	5·41	96·83	5·242
20	105,000	69·3	7·28	62·6	6·57	83·85	5·512
30	117,000	64·0	7·49	60·8	7·11	75·47	5·369

It is evident that the product $E \times D$ rises steadily with increase in E. In other words the value to be ascribed to D to maintain the product ED con-

stant would be one which would fall more rapidly with increase in alcohol content than is indicated by the values in Table VIII. Owing to the molecules of the medium being almost certainly present in an oriented unimolecular layer [Svedberg and Nichols, 1927] the dielectric constant of the actual medium in which the denaturation reaction occurs probably has a value of the order of 7 in pure aqueous solution [Rideal, 1930].

Correspondingly reduced values should have been employed in the calculation of the product $E \times D$.

It will be evident however that simple proportionality between change in dielectric capacity of the surface layer and that of the bulk of the solution in the case of alcohol-water mixtures is only to be anticipated if the surface adsorbability of the water and alcohol molecules were the same; a very unlikely assumption. If alcohol molecules are preferentially adsorbed, for example, the value to be ascribed to D should fall more rapidly than is indicated by the values employed in Table VIII. As a matter of fact it is found that the empirical relation $E \times D \times C_w$ is a constant, as is indicated by the final column of Table VIII, where C_w is the mass of water present in each alcohol-water mixture.

SUMMARY.

1. The speed of denaturation of haemoglobin has been determined in the presence of varying amounts of alcohol. The alcohol content was varied from 0 to 30 vols. %, the temperature from 30° to 60·5°, and the p_H from 5·19 to 7·74.

2. Unimolecular velocity constants were obtained throughout, and these constants increased in magnitude with increasing alcohol content under otherwise similar conditions.

3. It was found that the minimum point on the p_H-velocity curve was shifted considerably from the neutrality point of water to the acid side by addition of alcohol. The curve was no longer symmetrical about the minimum point, being more abrupt on the side of lower p_H values than on that of greater p_H values.

4. The critical increment of activation was found to increase appreciably with the amount of alcohol present. It was found that the magnitude of the critical increment was inversely proportional both to the dielectric constant of the medium and to the percentage by weight of water present.

This investigation was carried out under the direction of Prof. W. C. M. Lewis, to whom the author wishes to express his thanks for much helpful criticism and advice. The author also wishes to acknowledge a grant made by Imperial Chemical Industries, Ltd., to the Department of Physical Chemistry of the University of Liverpool.

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CLXXXIX. THE EFFECT OF LIGHT AND SALTS ON GELATIN.

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Although it has long been known that gelatin which has been treated with potassium dichromate becomes insoluble on exposure to light, the past workers investigating this problem have assumed that the protein molecule was unchanged and have concerned themselves only with the changes in the dichromate. The reactions involved in the production of insoluble gelatin by the action of chromium salts in the absence of light were also investigated on the same lines. The most important work on the changes occurring in the first process has been carried out by Eder [1925, 1926], while the changes involved in the second process have been chiefly investigated by Lumière and Seyewitz [1902, 1905, 1906].

As early as 1891 Eder showed that the action of light on a potassium dichromate-gelatin mixture was to produce chromium chromate. The next important investigation made by Eder was on the action of heat and moisture on dichromated gelatin in the absence of light. He found that if the solution was kept at the boiling point for 1 or more hours, water being added from time to time to make up for loss by evaporation, it lost its consistency, became thick, and solidified while still at a high temperature. This boiled gelatin, after drying, still gave a gel when immersed in cold water although more slowly and with greater difficulty than before. He also found that air-dried dichromated gelatin became insoluble when kept for a long time in the dark at the ordinary temperature, although much less readily than when in the form of a jelly. Returning to the action of light, Eder observed that dichromated gelatin which contained a trace of insoluble material became insoluble much more rapidly than that which was entirely unchanged. Finally he stated that the exposed parts of a dichromated gelatin film became more completely insoluble when subsequently kept in the dark, while the unexposed parts did not, and he considered that this after-effect could only be stopped by washing out the dichromate.

It must be remembered that throughout this work, Eder used ordinary photographic gelatin and some of the changes which he found may well be due to the presence of impurities such as minute traces of reducing agents, since, as will be shown later, immediate insolubility is produced by the

addition of a reducing agent to dichromated gelatin in the dark. As a chemical change of the type suggested will take place only in solution, this would account for the increase in the rate of change observed when the mixture was moist. That heating should hasten such a reaction is well known. The present writer in an attempt to reproduce some of Eder's results has used a highly purified gelatin and finds that when such gelatin is treated with potassium dichromate it will not become insoluble in the dark. Further, this pure gelatin was not rendered insoluble by boiling for 1 hour with potassium dichromate. It is therefore apparent that the changes which Eder found are due entirely to the presence of impurities in his material.

Lumière and Seyewitz [1905] investigated the composition of gelatin made insoluble by treatment with dichromate and exposure to light. They found that gelatin which had been rendered insoluble by the action of dichromate and light contained chromium sesquioxide and potassium oxide, the latter giving potassium chromate by reaction with excess of dichromate.

$$K_2Cr_2O_7 + K_2O = 2K_2CrO_4.$$

They repeated [1906] Eder's investigations on dichromated gelatin rendered spontaneously insoluble in the dark and found that insolubility was slowly produced at ordinary temperature but rapidly at 120° F. The present writer attributes these changes to the presence of impurities since Lumière and Seyewitz, like Eder, did not use purified gelatin. In no case do they mention the source and previous treatment, if any, of their gelatin, so the exact conditions prevailing in these experiments cannot be ascertained, but from experiments with pure gelatin to be described later it is clear that their results are incorrect.

Marion [1873] made the discovery that if a dichromated gelatin film which had become insoluble by exposure to light under a negative were placed in contact with a similar but unexposed film and the two were squeezed together under pressure between moist blotting paper, the gelatin in contact with the exposed gelatin became insoluble after 8–10 hours, and a print of the image was so obtained. It was concluded that once action was set up by light in a dichromated gelatin film this could induce the same change in gelatin placed in contact with it, without exposure to light. If this were the true explanation it seems remarkable that the outline of the image is preserved, since one would expect the change to occur in the unchanged material around the exposed parts. It seems more probable that the real change is purely mechanical, the squeezing causing the insoluble material to impregnate the soluble gelatin in contact with it, thus making it more difficult to dissolve. The present writer, therefore, attempted to reproduce this change in the absence of any mechanical influence and found that no such alteration occurred.

More modern workers on this problem are Schiel [1926] and Schommer [1926], but they have considered chiefly the changes involved in the bromol and ozobrome printing processes. Schommer considers that the oxygen

liberated during the ozobrome process is the important agent in producing insolubility. Liesegang [1926] however in a criticism of this work asserts that the reasons for assuming this are invalid. Further, since the addition of a reducing agent would produce immediate insolubility, it is clearly incorrect to attribute the change to the action of oxygen.

It can be seen from the foregoing that the results of past workers on the action of light on dichromated gelatin have really contributed little to our knowledge of the essential changes. They have shown that the dichromate is reduced and that chromium as the sesquioxide is fixed by the gelatin, but these workers have not investigated changes in the gelatin itself or shown whether such changes do occur. The present writer having pure gelatin at her disposal undertook the following investigations, repeating only such work on the changes in the dichromate as was necessary.

(1) Insoluble gelatin was prepared by three methods and the ash in several samples of each form was estimated. If insoluble gelatin were a definite compound all samples should contain the same percentage of ash. (2) The rate of the reaction proceeding when light acts on dichromated gelatin was investigated and this was compared with the rate of reaction when a known small amount of sodium sulphite was added to the mixture. These experiments also showed the maximum change obtainable and suggested the possibility of a reversible reaction. (3) The possibility of a reversible reaction was investigated. (4) The catalytic action of the insoluble material was investigated. (5) The Hausmann numbers of pure and insoluble gelatin were determined.

During the course of this research a new insoluble ash-free gelatin has been prepared. When acetone is added to a solution of gelatin or dichromate-gelatin in water the protein is precipitated, but the precipitate is soluble in water. If however the solution of dichromate-gelatin be first exposed to light, the precipitate obtained by adding the acetone will no longer dissolve in water. It is ash-free, consequently the change produced is not due to any combination of the gelatin with chromium. It is reasonable to suggest that the change is a denaturation, which is of great interest, for, as is well known, gelatin cannot be coagulated by heat and as far as the present writer is aware has not yet been obtained in a denatured form.

The results obtained have led to the conclusion that the first action of the light is to produce a change in the protein itself, either the denaturation referred to above, or a change preparatory to denaturation, which in any case involves no fixation of chromium. After this alteration the gelatin which is still in solution becomes insoluble either by fixation of chromium by prolonged exposure, or by keeping in absence of light for about 2 weeks, or immediately by precipitation with acetone.

The properties of this insoluble ash-free gelatin were investigated as follows.

(1) The Hausmann numbers were determined. (2) The rate of digestion of the insoluble gelatin by pepsin was determined and this was compared

with the rate of digestion of ordinary gelatin. (3) A dichromated gelatin solution was exposed and most of the dichromate was removed by dialysis after exposure. The optical rotation of the solution obtained was determined and was compared with that of a solution of pure gelatin.

Experimental.

The gelatin used was Coignet's Gold Label gelatin purified by electrodialysis and flocculation [Schryver, 1923]. The ash in the gelatin so prepared was less than 0·02 %.

Percentage of ash in insoluble gelatin prepared by three methods.

(1*a*) To a solution containing approximately 4 % gelatin, sufficient potassium dichromate was added to give a 3 % solution, and this was spread as a film on a glass plate and exposed to the light from a mercury vapour lamp for 2 hours. The insoluble material formed was scraped off the plate and washed with distilled water, first cold and finally at 30°, until the washings were colourless; after drying at 110° the ash amounted to 5·21 %.

(1*b*) The gelatin was treated as above, but after the washing with water it was washed with 1 % hydrochloric acid which removed a further quantity of dichromate; the acid was then removed by washing with distilled water, and the material was dried at 110°. The ash was 4·34 % in one experiment and 4·62 % in another.

(2) The gelatin was made insoluble by treatment with potassium dichromate and addition of sufficient powdered sodium sulphite to give a 0·5 % solution. The insoluble product was washed as in (1*b*) above and the ash determined. (*a*) 3·68 %, (*b*) 4·05 %.

(3) The gelatin was made insoluble by adding sufficient chrome alum to give a 3 % solution and allowing to gel. The product was washed and dried as before and the ash determined (3·93 %).

These results do not suggest the formation of a definite compound.

The rate of change of dichromated gelatin on exposure to light.

Potassium dichromate was added to a gelatin solution to give a 3 % solution of the salt. The exact concentration of the gelatin was found by nitrogen determination. A series of exposures of increasing duration was made with 2 cc. samples of this solution spread over the bottom of 100 cc. beakers. After the given time the unchanged gelatin was dissolved by washing first with cold water and finally with water at 30°, the solution was filtered and made up to 25 cc. By estimating the nitrogen in this solution the amount of unchanged gelatin was found. The results are given in Table I.

This rate of change was compared with the rate of formation of the insoluble products obtained by adding 0·2 cc. of a 5 % sodium sulphite solution to 2 cc. of the dichromated gelatin at the beginning of the exposure, the results of which are given in Table II.

Table I. *Rate at which dichromated gelatin was rendered insoluble by exposure to light.*

Time exposed (min.)	% changed	Time exposed (min.)	% changed
10	3·1	90	65·0
20	7·1	100	89·4
40	13·2	120	88·3
60	21·7	200	88·5
80	46·5	220	89·7

Table II. *Rate at which dichromated gelatin in the presence of sodium sulphite was rendered insoluble by exposure to light.*

Time exposed (min.)	% changed	Time exposed (min.)	% changed
2·5	7·0	10	66·4
5·0	16·0	15	79·2
7·5	46·4	20	85·1

Although the second change is far quicker than the first, it is significant that in both cases the curves constructed from the data given in Tables I and II are similar in shape, and also that the rate suddenly increases after about 20 % of the gelatin has been changed. The maximum change produced by a prolonged exposure with the sodium sulphite was found to be 88·8 %. This is in close agreement with the maximum change produced by simple exposure with dichromate, *i.e.* 89·7 %. The maximum change produced in dichromated gelatin in the absence of light by addition of sodium sulphite is 88·2 %; the maximum change produced by 3 % chrome alum is 89·0 %. That the residual nitrogen in each case is due to the presence of unchanged gelatin is shown by the fact that the filtrates give gels on evaporation, which can be made insoluble by exposure. It is obviously not possible to obtain a complete change to the insoluble form although former workers have made no mention of this fact.

To determine whether the reaction is reversible.

From the above results it seemed probable that the reaction was reversible, equilibrium being attained when about 90 % of the material had become insoluble. To determine this, known weights of the insoluble gelatin, which had been well washed with water and dried in a vacuum desiccator over sulphuric acid, were moistened with water and exposed for one hour to the mercury vapour lamp. After exposure they were well washed with water and the nitrogen in the washings was determined.

In the first experiment 0·106 g. of insoluble material, containing 0·006 g. of chromium trioxide and adhering dichromate was used. The filtrate contained 0·34 mg. N, corresponding to 2·0 mg. of gelatin. In the second experiment the insoluble material amounted to 0·172 g. and contained 0·009 g. of the chromium trioxide and dichromate. The filtrate contained 0·52 mg. N, corresponding to 3·3 mg. of gelatin, *i.e.* approximately 2 % of the gelatin had gone into solution.

If the nitrogen in the filtrate really represents gelatin which has become soluble, these results would suggest that the action is slowly reversible; but the amount of material in the filtrate is too small to give a gel, and although a precipitate is obtained by adding acetone, other tests showed the presence of amino-acids only and did not confirm the presence of a protein. The reversibility of the reaction was therefore not definitely proved.

Catalytic action of the insoluble material.

Since the rate of formation of the insoluble compound increases with time the effect of the addition of minute traces of the insoluble product was investigated. When 0·05 g. of the insoluble gelatin was exposed with 2 cc. of an approximately 4 % solution of dichromated gelatin, the extent of the solution of the insoluble form completely masked the reverse change occurring in the 2 cc. of soluble gelatin. To overcome this, 2 cc. of dichromated gelatin of known concentration was exposed for 2 hours to insure complete insolubility, after which it was well washed and the nitrogen in the filtrate was determined. A similar exposure was made with another 2 cc. sample but after exposure a further 2 cc. was added to this and the exposure was continued for 1 hour. The product was washed with water, filtered and the nitrogen in the filtrate was determined. The ammonia derived from 2 cc. of the gelatin solution in a Kjeldahl determination neutralised 57·5 cc. of $N/70$ acid. Exposure for 1 hour rendered insoluble gelatin equivalent to 15·5 cc. of acid or 27 % of the total. When 2 cc. of the gelatin after exposure for 2 hours was added to another 2 cc. and the whole was exposed for another hour, the nitrogen in the filtrate was equivalent to 39·5 cc. The gelatin which had been exposed for 2 hours contributed to this only 5·0 cc., so that the 2 cc. of gelatin exposed for only 1 hour—but in the presence of gelatin already rendered insoluble—contributed 34·5 cc., showing that an equivalent of 23·0 cc. or 40 % of the gelatin had been rendered insoluble. These changes definitely prove the catalytic action of the insoluble material. That the catalysis was not due to the small amount of gelatin which remained soluble was proved by the inability of this fraction to catalyse the reaction after it had been separated from the changed material.

Repetition of Marion's experiments in the absence of any mechanical influence (*see p. 1707*).

A known amount of dichromated gelatin was exposed until maximum insolubility had been produced, an equal amount of the same gelatin was added and the whole was left in the dark for 24 hours. After this time the mixture was washed and the nitrogen in the filtrate was determined. It was seen from this that no change had occurred in the added gelatin. The experiment was repeated but the mixture was allowed to remain in the dark for a greater length of time; again no change was found. The results obtained were as follows.

		N (cc. $N/70$ acid)
2 cc. of the gelatin used	contained	18·5
Soluble fraction from 2 cc. of this gelatin after exposure for 3 hours	„	2·0
Filtrate from 2 cc. of gelatin exposed for 3 hours and left for 24 hours with an additional 2 cc. of gelatin in the dark ...	„	20·5
Above mixture after leaving for 2 weeks	„	20·3
Above mixture after leaving for 4 weeks	„	20·4

Determination of Hausmann numbers.

The Hausmann numbers of pure gelatin, gelatin made insoluble by exposure with dichromate, and insoluble ash-free gelatin were determined by the micro-method of Thimann [1926]. The latter has since shown [1930] that in order to obtain results truly comparable with one another the estimations should be carried out on solutions containing equal concentrations of nitrogen, since the percentage of nitrogen precipitated by phosphotungstic acid varies continuously with the total concentration of nitrogen. Several estimations were therefore made with the three forms of gelatin and curves were constructed showing the relation between the percentage of nitrogen precipitated by phosphotungstic acid, and the concentration of nitrogen in the solution used. The values for pure gelatin agreed closely with the values for the insoluble ash-free gelatin, but were in all cases 1–2 % higher than the values obtained with the gelatin made insoluble by exposure; since it is known that the presence of salts lowers the percentage of nitrogen precipitated by phosphotungstic acid the estimations with pure gelatin were repeated, but sufficient potassium dichromate was added to the hydrochloric acid used for the hydrolysis to correspond approximately with the chromic oxide present in the insoluble product. The values obtained for the two forms were then the same, as shown by the results in Table III.

Table III. *Phosphotungstic acid precipitates from hydrolysis products of pure gelatin and insoluble dichromated gelatin after hydrolysis with 20 % HCl for 20 hours.*

Pure gelatin + 5 % $K_2Cr_2O_7$		Insoluble dichromated gelatin	
Total N (cc. $N/70$ acid)	% of total N in phosphotungstic acid precipitate	Total N (cc. $N/70$ acid)	% of total N in phosphotungstic acid precipitate
54·0	19·5	20·5	15·9
85·2	21·7	59·0	21·0
130·0	21·0	89·7	21·6
159·2	19·5	141·8	20·9
		152·8	20·0

From the curves constructed it was seen that the change which occurs in the protein molecule whereby it is rendered insoluble is not one which can be detected by an examination of the Hausmann numbers.

Table IV. *Phosphotungstic acid precipitates from hydrolysis products of pure gelatin and insoluble ash-free gelatin.*

Pure gelatin		Insoluble ash-free gelatin	
Total N (cc. $N/70$ acid)	% of total N in phosphotungstic acid precipitate	Total N (cc. $N/70$ acid)	% of total N in phosphotungstic acid precipitate
31·5	19·6	25·2	18·4
42·0	20·15	42·0	20·0
50·1	21·0	93·4	23·1
87·5	23·2	121·0	23·0
139·5	22·9		

Preparation and properties of the insoluble ash-free gelatin.

A solution of dichromated gelatin was exposed to the light of a mercury vapour lamp for 2 hours, and was stirred periodically to break up the insoluble film which formed on the surface. After the solution had been filtered from the insoluble particles, acetone was added to precipitate the protein and the mixture was left to stand overnight. The precipitated material was then filtered off and well washed with water to remove any unchanged gelatin and adhering potassium dichromate; it was then dried, first by standing in acetone overnight and finally in a vacuum desiccator over sulphuric acid. This gelatin, after drying, is opaque and greyish white in colour and can be ground to a coarse powder. A whiter and finer preparation may be obtained by allowing the gelatin to stand in water for several weeks before the final drying. The material swells but will not dissolve in either 1 % hydrochloric acid or glacial acetic acid even on warming to 40°. It is insoluble in a solution of sodium salicylate which is a particularly good solvent for ordinary gelatin. The ash amounts to 0·2 % which shows that in this gelatin there is no fixation of chromium. The Hausmann numbers are the same as for ordinary gelatin as shown in Table IV. The rate of digestion by pepsin is the same as for ordinary gelatin as shown by the following experiment.

Rates of peptic digestion of pure and insoluble gelatin.

In these determinations, Willstätter's method was used for the estimation of the liberated amino-acids. Both the insoluble and pure gelatin were used in the granular form obtained by drying in a vacuum desiccator over sulphuric acid after precipitation with acetone. 0·5 g. of material was placed in a 50 cc. conical flask and to this was added 5 cc. of distilled water followed by 5 cc. of an 8 % solution of pepsin in hydrochloric acid. The gelatin was incubated at 20° and after incubation the mineral acid was neutralised by $N/4$ sodium hydroxide, using litmus as an external indicator. The solution was then transferred to a 250 cc. conical flask and to it was added 0·5 cc. of a solution of 0·5 % phenolphthalein in absolute alcohol. The amino-acids were then estimated by titration with $N/25$ alcoholic potassium hydroxide, a further 20 cc. of alcohol being added each time the solution became pink until 100 cc. of

alcohol had been added. The solution then contained approximately 90 % of alcohol which is that required to give the true end-point. The results obtained showed no difference between the two forms, as shown in Table V.

Table V. *Peptic digestion of pure and insoluble ash-free gelatins.*

	Pure gelatin		Insoluble ash-free gelatin	
Time of incubation (hours)	cc. *N*/4 NaOH	cc. *N*/25 alcoholic KOH	cc. *N*/4 NaOH	cc. *N*/25 alcoholic KOH
1	2·00	18·0	2·08	18·5
2	2·06	19·0	2·10	19·0
4	2·16	20·5	2·15	20·0
24	2·20	22·5	2·18	22·0
48	2·20	23·5	2·20	23·0

To investigate the possibility of racemisation in insoluble gelatin.

To determine whether racemisation had occurred the optical rotations of solutions of pure gelatin and of gelatin which had been exposed with dichromate were examined. Before using the latter solution the greater part of the potassium dichromate was removed by dialysis. The exact concentrations of the solutions were found by nitrogen estimations. The value for the rotation of exposed gelatin was slightly lower than for the pure material, but the differences obtained were too small to allow of the definite conclusion that racemisation had occurred. The use of more concentrated solutions would, no doubt, have given more reliable results but was found to be impossible owing to opacity. The rotations of the products of hydrolysis of pure gelatin and of ash-free insoluble gelatin were next determined. The material was hydrolysed by boiling for 20 hours with hydrochloric acid and the acid was then evaporated under reduced pressure. The residue was taken up in water, and the solution was clarified by boiling with a little animal charcoal, filtered and made up to a known volume. Before measuring the rotations, the solutions were made alkaline by the addition of 10 cc. of 10 % caustic soda to 20 cc. of solution. In the solutions so obtained no difference in rotation was observed.

Conclusions.

From the experimental results quoted above it is possible to draw the following conclusions.

When gelatin is made insoluble by treatment with dichromate and exposure to light, a change in the protein, allied to denaturation, first takes place but this involves no change in the Hausmann numbers or in the digestibility of the protein. Chromium is then fixed in the form of the sesquioxide but a definite compound is not involved.

The reaction does not proceed to completion and is probably reversible, equilibrium being attained when 90 % of the gelatin has become insoluble.

The reaction is catalysed by the insoluble gelatin.

From the preparation of the insoluble ash-free gelatin, it can be clearly

seen that coagulation is preceded by a change which produces no alteration in the physical state of the solution. This confirms the suggestion first made by Hardy [1899] and supported by Chick and Martin [1910] that coagulation takes place in two stages, the first being a chemical reaction, called by Chick and Martin "denaturation," and the latter, which produces the coagulation, merely physical. In the case of gelatin the denatured protein can be definitely and completely isolated from the coagulated product, and the existence of such a form in the case of gelatin does much to support the theory of denaturation in other proteins.

To the late Prof. S. B. Schryver, under whose direction the work was started, the writer is most deeply indebted.

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CXC. ON THE PRESENCE OF A VOLATILE ORGANIC CHLORINE COMPOUND IN BLOOD.

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It is generally assumed that chlorine exists in the blood solely as inorganic chloride. While this hypothesis is supported by a great deal of the classical work of L. J. Henderson, Van Slyke and others on the interchange of ions during disturbance of acid-base equilibrium, it is nevertheless true that some problems are not solved by such a conception. During a prolonged investigation into changes in the chemistry of the blood in pyloric stenosis of infancy, it has become evident that the loss of chlorine from the blood is not necessarily balanced by the gain in carbonic acid or other known acid [Graham and Morris, 1929]. Further as Hanke and Donovan [1927] state "The current theories (of the formation of gastric hydrochloric acid) involve the unreasonable assumption of the separation of the acid locally from an equivalent of alkali." These authors maintain that 10–50 % of the total chlorine is present in organic form in the blood and tissues. They suggest that it is by the action of a special chloro-esterase on this organic chlorine compound that the free HCl of the gastric juice is produced.

The work reported in this paper was commenced at the suggestion of Prof. Leonard Findlay in an endeavour to throw some light on the nature of the chlorine combination in the blood. The method of chlorine analysis employed was that of Van Slyke [1923]. The subjects from whom the blood was obtained were healthy adults or children apparently completely recovered from such conditions as rheumatism. In all cases the subject had ingested nothing for a period of at least 12 hours prior to the withdrawal of the blood.

Effect of drying on the chlorine content of blood-serum.

After a preliminary drying of serum it was found that the amount of chlorine recovered was less than when no drying was employed. In each case to obtain the effect of drying the serum was heated in an Erlenmeyer flask in an oven at 105° for 45 minutes. Table I shows the results obtained from the sera of normal adults.

Table I. *Effect of drying on the chlorine content of serum.*

Serum No.	Chlorine milli-eq./litre	
	No drying	Preliminary drying
1	102·11	97·65
2	100·00	95·10
3	102·39	98·24
4	104·76	100·32

The loss of chlorine on drying might be due to one of four causes: (1) the presence of free chlorine in the blood which was liberated by some complex action similar to that of porous clay on NaCl; (2) the formation and volatilisation of HCl; (3) the loss of inorganic chloride by volatilisation; (4) the presence of volatile organic chlorine.

The presence of free volatile Cl or HCl is excluded by the following experiments. The gases from 100 cc. of blood heated to 105° when led into a solution containing KI and starch failed to give a blue colour. The amount of Cl which is lost during the drying of 100 cc. of blood is sufficient immediately to produce a blue colour with starch iodide. The absence of this reaction together with the fact that no precipitate was found on distilling into silver nitrate solution indicates that only mere traces of free Cl or HCl could have been present.

Table II. *Effect of drying on the chlorine and fixed base content of serum.*

Serum No.	Chlorine milli-eq./litre		Fixed base milli-eq./litre	
	No drying	Preliminary drying	No drying	Preliminary drying
5	106·5	102·2	147·7	147·5
6	110·3	106·7	153·1	153·4
7	103·2	100·0	149·9	149·8
8	102·9	98·3	150·1	150·2
9	99·4	95·7	151·9	152·0

In order to determine the possibility of loss of inorganic chlorides the fixed base content of the serum was determined before and after drying by the method of Stadie and Ross [1925]. Table II gives the results. It is evident that the fixed base is unaltered by preliminary drying while definite loss of chlorine results from this treatment. Accordingly, inorganic chloride could not have been lost during the drying. One is therefore forced to the conclusion that the chlorine lost by drying is in the form of a volatile organic chlorine compound.

The effect of drying on the chlorine content of whole blood and serum was then estimated in order to determine the distribution of the volatile organic chlorine between serum and corpuscles. Blood was collected under oil and defibrinated; samples of whole blood, serum and cells were then analysed. The percentage cell content was determined by the haematocrit. Table III gives the results, from which it is evident that the cells contain a much greater amount of volatile organic chlorine than does the serum.

Table III.

Blood No.	Red blood corpuscles %	Chlorine milli-eq./litre: Whole blood, No drying	Whole blood, Drying	Serum, No drying	Serum, Drying	Cells, No drying	Cells, Drying	Organic volatile chlorine milli-eq./litre: Blood	Serum	Cells
10	45	78·57	65·47	101·56	96·82	49·77	24·58	13·10	4·74	25·19
11	46	80·11	65·88	100·48	96·04	55·58	25·10	14·23	4·44	30·48
12	46	79·95	66·15	102·15	98·10	54·06	24·82	13·80	4·05	29·24
13	44	78·99	66·55	102·76	97·85	48·43	25·77	12·44	4·91	22·66
14	45	79·15	66·90	101·09	97·14	52·44	28·77	13·25	3·95	23·67

Effect of varying the duration of the drying process on the organic volatile chlorine.

It is important that the drying process should proceed for at least 400 minutes in order to ensure the volatilisation of the chlorine to a maximum. The following results give proof of this.

Duration of drying in minutes	Volatile organic chlorine in milli-eq./litre
3	3·20
15	3·25
30	4·25
45	6·25
60	7·85
75	8·55
90	9·24
105	9·50
210	11·15
400	12·60
600	12·60

The isolation of the volatile organic complex of chlorine has so far been found impossible owing to the volatilisation of various substances at the same time. On distillation acetic acid and ammonia appear in very large amounts. Distillation at 110° accompanied by the passage of an air current through the blood led to the collection of but minimal amounts of chlorine in the receiving flask. By the usual distillation procedure, however, it was possible to recover and estimate by the Carius or Parr method the amount of Cl volatilised; Table IV gives the amounts so recovered. The amounts collected in the distillates compare very satisfactorily with those lost from the blood samples especially when one remembers the practical difficulties which involve a large experimental error such as the possibility of some of the volatile chlorine being held back by the material as it dries.

Table IV. *Chlorine lost from the blood on distillation.*

Blood No.	Chlorine milli-eq./litre: Lost from blood	Collected in distillate: Parr method	Collected in distillate: Carius tube
15	172·4	168·0	165·1
16	146·5	142·1	144·3
17	141·8	140·1	140·7
18	138·7	132·4	136·2

Effect of addition of various substances on the volatile organic chlorine content of blood.

Various compounds containing the NH_2 group were used, including urea, histamine, glycine and a few of the simpler primary and secondary amines. In all cases the materials employed were purified before using. In Fig. 1 is

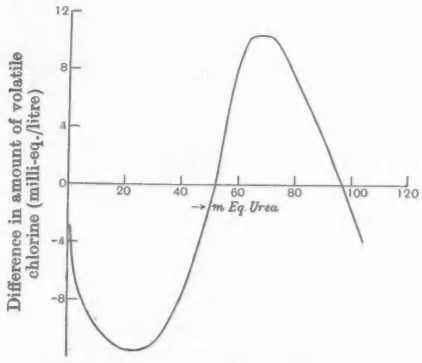

Fig. 1. Effect of urea-concentration on volatile chlorine.

given a graphic representation of the results obtained when urea was added to the blood in progressively increasing amounts.

To a sample of blood a known amount of urea was added: the two portions were then analysed (one with and the other without previous drying) to obtain the value for the volatile chlorine. All analyses were done in duplicate. It will be seen that this concentration curve corresponds with the adsorption formula $y/m = kc^{1/p}$, where y = amount adsorbed by a quantity m of adsorbent, c = concentration at equilibrium and k and p are constants. In the present case y represents the increase in urea concentration, m the original amount of volatile chlorine and c the amount of volatile chlorine after the addition of the urea. Between the concentrations of urea 2·14–24·10 milli-eq./litre the values of $1/p$ and k are $-3{\cdot}4$ and $-3{\cdot}16$ respectively. There is a marked resemblance between the curve and the coagulator concentration curves. In this case the coagulator is urea and the reaction consists of a coagulation of the volatile chlorine complex up to a range of concentration of urea of 24 milli-eq., when reversal occurs with consequent peptisation.

When an ammonium salt is added, *e.g.* $(NH_4)_2SO_4$, a curve similar to that of the NH_2 series is obtained (Fig. 2). The maximum and minimum values (*i.e.* those concentrations at which reversal takes place) are lower than those found with urea. The curve again corresponds with the adsorption equation. Between concentrations of $(NH_4)_2SO_4$ of 0·9672–4·8360 milli-eq./litre $1/p = 2{\cdot}4$ and $k = -12{\cdot}13$.

When a chloride is added, the results obtained are the inverse of those

obtained with NH_2 or NH_4 groups (Fig. 3). This indicates that the addition of the chlorine ion produces an increase in the amount of volatile chlorine, possibly because the Cl having the opposite electric charge to that of NH_2 breaks the union of NH_2 and volatile chlorine. At a certain concentration reversal sets in with the consequent diminution of volatile chlorine.

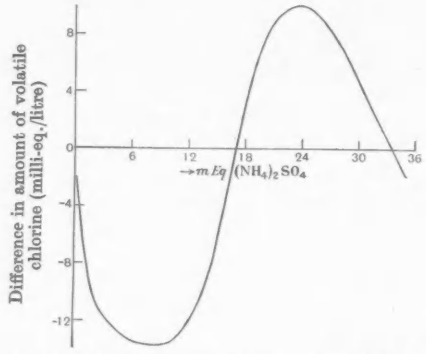

Fig. 2. Effect of NH_4 content on volatile chlorine.

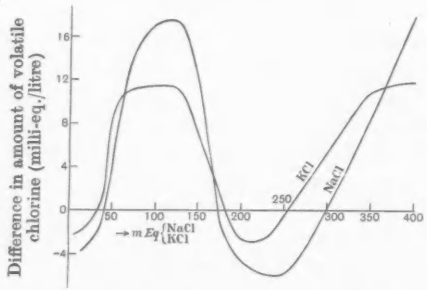

Fig. 3. Effect of KCl and NaCl content on volatile chlorine.

Addition of a very dilute acid produces the same effect on volatile chlorine as does NaCl. As the strength of acid is increased, the amount of volatile chlorine is at first raised and then lowered till with higher concentrations free HCl is formed.

Effect of prolonging the time during which blood was allowed to stand at room-temperature.

The mixtures of blood and coagulator substances (urea or NaCl) were allowed to stand at room-temperature before drying. The mixtures had urea 1·605 and NaCl 50 milli-eq./litre respectively. The results are given in Table V. The general equation for both curves is $y = \alpha c^n$. For the urea mixture $\alpha = 0{\cdot}54$, $n = 1{\cdot}7$ and for the NaCl mixture $\alpha = 44$, $n = -0{\cdot}15$. The effect on

Table V. *Effect of time-factor on volatile organic chlorine of blood to which urea or NaCl has been added.*

Time in minutes	Difference in amount of volatile organic chlorine in milli-eq./litre Blood urea	Difference in amount of volatile organic chlorine in milli-eq./litre Blood NaCl
5	−4·1	−5·7
10	−4·2	−5·46
15	−4·88	−4·62
20	−5·76	−3·58
25	−7·08	−2·56
30	−8·05	−1·56
40	−8·90	−0·46

the volatile organic chlorine of allowing untreated blood to stand was to cause a gradual decrease to a value which remained steady about 5 milli-eq. below the original. Thereafter prolonged standing did not appreciably alter the amount of volatile chlorine. This is interesting in view of the results of Havard and Kerridge [1929] on the change of c_H in blood allowed to stand. They found that the c_H increased to a point and there remained steady. It has already been pointed out that the addition of acid in small amount decreases the content of volatile chlorine so that the effect of standing on the volatile chlorine is associated with the increase in c_H. It seems feasible to assume that the liberation of volatile chlorine produces an increase in the c_H. It is possible however that the relationship of cause and effect is reversed or that loss of volatile chlorine and increase in c_H are both due to some other underlying factor.

From these data we may conclude that the amount of volatile chlorine may be altered by the addition of various substances to the blood but that for each blood there is a certain maximum and minimum beyond which the content does not pass. The simplest hypothesis on which to explain these results is to assume that the chlorine in normal blood exists in three forms (*a*) inorganic, (*b*) organic non-volatile, and (*c*) organic volatile. The relative amounts of non-volatile and volatile organic chlorine seem to depend on the concentration of urea, amino-acid etc. Thus in blood I (Fig. 1) the maximum amount of volatile chlorine is 24·79 milli-eq. per litre. Varying amounts of urea lead to the production of an organic non-volatile chlorine compound in amounts that satisfy the adsorption formula. This indicates the presence of a complex organic colloidal system in which there is present a loose union of amino-compounds with chlorine forming a non-volatile organic compound. As the amino-compounds are increased in amount there occurs an increased formation of this non-volatile compound up to the point where reversal of charge sets in when peptisation occurs. The addition of an inorganic chloride breaks this complex up to a certain concentration of the salt beyond which the chlorine once again commences to unite with the amino-group. This is probably also an effect of the electric charge.

We wish to express our thanks to the Medical Research Council for defraying the expenses of the investigation and for a personal grant to one of us (N. M.).

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CXCI. THE ACTION OF ARSENITE ON TISSUE RESPIRATION.

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The action of arsenious acid on respiration was studied first by Onaka [1911]. This author found that arsenite, like cyanide, strongly inhibits the oxygen uptake of avian red blood corpuscles. More recently Dresel [1926] found the oxygen uptake of rat liver to be inhibited by arsenite, while the formation of lactic acid by Jensen's sarcoma was but little affected by this poison. Both the fermentation and oxygen uptake of yeast were found to be greatly inhibited by arsenite. No attempts were made to determine more closely the part of the oxidation system on which arsenite exerts its influence. Onaka's results suggested that the mode of action was identical with that of cyanide which is known to inactivate the indophenol oxidase (Atmungsferment, cytochrome oxidase).

Rabbit's liver was used as material in the present investigation. The oxygen uptake was measured in the Barcroft apparatus. The rates of respiration with and without arsenite or cyanide respectively are given in Table I. As the table shows the respiration is inhibited to a similar (large) extent by both poisons.

Table I.

	O_2 uptake in 10 min. ($mm.^3$)			Mean
Liver (1 g.)	240	293	218	284
" +0·01 *M* KCN	86	88	106	93
" +0·002 *M* As_2O_3	99	112	67	93
% inhibition by KCN	64	70	67	67
" As_2O_3	59	62	79	67

Table II.

	% inhibition of O_2 uptake by liver pulp	
As_2O_3 (*M*)	1st 10 min.	2nd 10 min.
0·01	80	86
0·0045	76	85
0·002	77	80
0·0009	75	80
0·0004	71	79
0·00013	50	68
0·00004	16	25

[1] Fellow of the Rockefeller Foundation.

Table II gives the inhibition by different concentrations of arsenite. It will be seen that even very high dilutions have a strong depressing action and that the inhibition tends to reach a maximal value at a very small concentration and then to remain roughly constant over a wide range of dilutions. In both respects arsenite resembles cyanide [Dixon and Elliot, 1929].

The difference of the mechanism of action of cyanide and arsenite can however be demonstrated very clearly by the behaviour of the indophenol oxidase. If the minced liver is washed with water and then suspended in phosphate in presence of *p*-phenylenediamine the system shows a strong oxygen uptake owing to the oxidation of the phenylenediamine by the indophenol oxidase. This enzymic action, which represents the so-called oxygen activation, is entirely inhibited by small concentrations of cyanide, but is quite insensitive even to the highest (0·01 *M*) concentrations of arsenite (Table III).

Seeing that the arsenite had no effect on the oxygen activation it seemed probable that it poisoned the hydrogen activation. This latter mechanism can easily be observed in the oxidation of succinic acid. As shown by Fleisch [1924] and also by myself [1924] activation of both hydrogen and oxygen is required for the oxidation of this substance. If either of these activating mechanisms is impaired the oxygen uptake stops. As shown in Table II the oxidation of succinic acid is relatively insensitive to high concentrations of arsenite[1].

These experiments led therefore to the conclusion that arsenite does not inhibit respiration by poisoning the activation either of hydrogen or of oxygen. According to our present knowledge of the mechanism of oxidations in tissues these observations suggest that the arsenite interferes with some reaction or reactions which take place prior to the hydrogen activation, in which the metabolites are prepared by anaerobic changes for their oxidation. It is generally held by biologists that oxidations are always preceded by anaerobic changes, which are usually regarded as fermentation. It seems probable that arsenite interferes with one or more of these reactions, and inhibits the respiration by inhibiting the elaboration of dehydrogenisable, *i.e.* combustible, substances.

The behaviour of fresh unwashed liver tissue towards methylene blue under anaerobic conditions is in good agreement with this conclusion. If the freshly minced liver is suspended in phosphate and methylene blue is added *in vacuo*, the dye is rapidly decolorised owing to the oxidation of metabolites accumulated during the manipulations or formed at the time of the experiment. If the suspension is kept in contact with arsenite prior to its incubation with methylene blue, the reduction time will be found to be 25 % longer, owing apparently to the slower production of dehydrogenisable substances during the experiment. If prior to the addition of arsenite the suspension is

[1] Citric and lactic acid were used in an attempt to get additional evidence, but it was found that both substances were oxidised by washed liver too slowly to give reliable results.

incubated anaerobically, time being given for metabolites to accumulate, the reduction time is found to be but slightly increased by arsenite. This shows that the latter has but little effect on the rate of dehydrogenation of natural hydrogen donators. If on the other hand the suspension is aerated, time being given for the metabolites already formed to oxidise, the increase in reduction time in presence of arsenite becomes considerable (280 %).

It seemed interesting to compare these results obtained on liver with the action of arsenite on the respiration of yeast. It was found that the oxygen uptake of yeast can also be greatly inhibited by arsenite, but much higher concentrations of the poison are required[1] (Table IV).

The alcoholic fermentation (as measured by the CO_2 production) is practically unaffected by 0·057 *M* arsenite, which concentration almost completely inhibits the oxygen uptake (Table IV).

Table III.

	O_2 uptake in 10 min. (mm.³)	
Liver (0·8 g.) + succinate	368	346
" " + As_2O_3	356	324
" + *p*-phenylenediamine	90	175
" " + As_2O_3	87	182

Table IV.

Yeast (mg.)	As_2O_3 (*M*)	% inhibition	
		O_2 uptake	CO_2 output
100	0·06	90	4
100	0·03	49	0
100	0·003	44	0
100	0·0003	0	0

Arsenite thus gives a further method for the separation of respiration from alcoholic fermentation in yeast. The results are at variance with those obtained by Dresel [1926]. They are in accordance with the view of Lundsgaard [1930], who was led, by the study of the action of iodoacetic acid, to the conclusion that the processes involved in respiration and alcoholic fermentation are at least partly different. While iodoacetic acid separates respiration from alcoholic fermentation by inhibiting the latter, arsenite makes a separation possible by inhibiting the former.

Experimental.

The liver of the freshly killed rabbit was freed from the big veins and the gall bladder and minced in a machine to a fine pulp, which was kept at 0°. Portions of 1 g. were weighed out on watch glasses. The pulp was washed with 1 cc. of phosphate into the flask of the Barcroft apparatus of the usual type as described by Dixon and Elliott [1930]. 0·04 *M* phosphate was

[1] This difference might be due to a smaller permeability, though identical results were obtained with "plasmolysed" yeast.

used, prepared by mixing 2 parts of 0·04*M* Na_2HPO_4 with 1 part of 0·04*M* KH_2PO_4. 0·5 cc. of 0·05*M* As_2O_3 or 0·1*M* KCN was added. The middle tube was filled with 0·5 cc. of strong potassium hydroxide solution absorbed on filter paper [Dixon and Elliott, 1930]. In the compensating vessel the liver was replaced by 1 cc. of water.

The flasks were connected to the manometer and immersed in a water-bath at 40°. Shaking was immediately begun. After 10 min. the taps were closed, and readings were taken after 10, 20 and 30 min. The results are given in Table I.

In the experiments with *p*-phenylenediamine and succinic acid the liver pulp was suspended in 10 times its own volume of distilled water, the suspension was gently stirred for 10 min., and then centrifuged. The centrifugate was reduced in the mortar with sand to a macroscopically homogeneous pulp. This was suspended in phosphate buffer, 2 cc. being used per g. of washed liver pulp. The suspension was filtered through fine muslin, and 2 cc. of the filtrate were introduced into the Barcroft apparatus together with 0·5 cc. of 0·05*M* As_2O_3 and 1 cc. of 3 % neutralised *p*-phenylenediamine or 5 % sodium succinate. In the compensating vessel the liver suspension was replaced by phosphate. Without succinate or phenylenediamine the suspension showed practically no O_2 uptake.

In the experiments with methylene blue the freshly minced liver was suspended in phosphate buffer as before. To one part of this suspension 1/20 vol. of 0·056*M* As_2O_3 was added and to the other part 1/20 vol. of water. Both suspensions were left at 0° for 10 min., to give time for the arsenite to diffuse into the cells. 3 cc. of the liver suspension were introduced into Thunberg tubes, and there mixed with 1 cc. of 1:5000 methylene blue; the tubes were evacuated and incubated. Reduction time with arsenite 132 sec. and 145 sec.; without arsenite 105 sec. and 115 sec.

Part of the suspension was incubated without arsenite *in vacuo* for 30 min. Then the suspension was divided into two parts. To one part 1/20 vol. 0·056*M* As_2O_3 was added and to the other part an equal volume of water was added. Both were left for 10 min. at 0°, and then samples of 3 cc. were taken and introduced into Thunberg tubes with methylene blue as above. Time of decoloration of sample with As_2O_3 195 sec. and 205 sec.; without As_2O_3 180 sec. and 180 sec.

If prior to their incubation the suspensions were aerated by gently shaking them in the water-bath for 10 min. in big open flasks the reduction times were the following; aerated without As_2O_3 2 min. 55 sec. and 2 min. 45 sec.; with As_2O_3 6 min. 30 sec. and 6 min. 30 sec.

In experiments with baker's yeast 10 g. of yeast were suspended in 100 cc. of 0·01*M* buffer containing Na_2HPO_4 and KH_2PO_4 in the ratio 1:4. 1 cc. of this suspension was introduced into the Barcroft apparatus together with 1 cc. of 5 % glucose and 1 cc. of As_2O_3 solution was added. The compensating vessel contained in place of yeast only buffer solution. If O_2 uptake was

measured the middle tube was filled with paper soaked in strong potassium hydroxide. The Barcroft apparatus was immersed in the water-bath at room temperature (18°). Taps were closed 10 min. later. Readings were taken after 10 min. In experiments in which the CO_2 production was measured no potash was placed in the middle tube. 0·3 cc. of 0·1 *M* KCN was added to the suspension in order to exclude the Pasteur reaction. Corrections were made for the oxygen uptake. The results are given in Table IV.

Summary.

Arsenite strongly inhibits the respiration of minced liver tissue, but has no effect on oxygen activation, and but little effect on hydrogen activation. In yeast arsenite in higher concentrations inhibits respiration but has no effect on alcoholic fermentation.

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CXCII. THE EFFECT OF COOKING ON THE DIGESTIBILITY OF MEAT.

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The question of the effect of cooking on the digestibility of foods has long been a subject of discussion. General opinion may be summed up in the words of Hutchison [1927]: "It is an error to suppose that cooking increases the digestibility of foods. That is only true of vegetable foods. The digestibility of animal foods is diminished rather than increased by cooking."

This diminution of digestibility in cooked foods is upheld by Clementini [1923] who found that when animals were fed on autoclaved meat the nitrogen balance became negative. He states however that this may partly be accounted for by the dislike shown to the autoclaved meat by his experimental animals.

Richet, Oxner and Richards [1925, 1926] state that cooked meat is of less value as a food for fish than raw meat, since fish fed on cooked meat die about the fiftieth day whilst those kept on raw meat survive. It seems possible since meat formed the sole diet in these experiments that an alteration in the vitamin content may be one of the causes of the inferiority of cooked as compared with raw meat as a fish food. A similar observation was made on the diet of Eskimos by Heinbecker [1928] who states that the Eskimo in his natural state eats practically only raw flesh and remains healthy. On the contrary it is well known that when, owing to the spread of civilisation, the Eskimo cooks his meat, scurvy breaks out.

Waterman and Finks [1920] found that rats fed on cooked phaseolin showed better growth than those fed on raw phaseolin, and Waterman and Johns [1921], continuing the work *in vitro*, found more amino-N in a digest from cooked phaseolin than from raw. They digested the protein first with pepsin and then with trypsin in order to imitate conditions in the animal body as closely as possible. This greater rate of digestion with cooked protein has been confirmed by Wallen-Lawrence and Koch [1930], who found that heat-treated milk was more rapidly digested than raw milk owing to an alteration in the whey. As is pointed out in this paper, this falls into line with other experiments on the anti-enzymic action of raw animal tissues. Hahn [1897] showed that normal blood-serum resisted proteolytic digestion more than

heated serum, and Wells [1906] found that the rate of autolysis of tissues in heated serum exceeded that in unheated serum.

In view of these findings and since raw meat is so often recommended in cases of weak digestion, whilst re-cooked meat is condemned as very indigestible, a series of experiments was carried out with meat cooked in various ways to compare the relative digestive rates.

Experimental.

Since in the normal body tryptic digestion follows on digestion with pepsin, and since the hydrolysis of protein by trypsin is more complete if carried out subsequently to peptic digestion [Cole, 1928], it was thought desirable to use both enzymes in one series of experiments. In a second parallel set, trypsin alone was used to digest the meat in an endeavour to find if cooking affected the action of one or both enzymes. The rate of hydrolysis of protein was estimated by Sørensen's formalin titration method.

In any series of experiments a large and, as far as possible, homogeneous joint was divided into four portions. One was kept raw, two were cooked so that they ceased to be purple inside and became the bright red of underdone though cooked meat; a fourth was over-cooked so that it became a uniform brown throughout its thickness. One of the correctly cooked pieces after cooling was re-heated in the oven till it also was over-cooked to the same degree as the fourth piece.

The joints were allowed to cool, any obvious fat and connective tissue were removed and the remaining protein was finely minced. 75 g. of this mince were placed in a flask, 150 cc. distilled water were added and the whole was placed in an ice-chest overnight. The next day in the trypsin experiments 150 cc. of 0·8 % Na_2CO_3 were added and the flasks were put in a water-bath at 37°. They were left 60 minutes for the flask contents to rise to the bath temperature, when 20 cc. of 10 % solution of pancreatic substance were added. The mixture was shaken, 20 cc. were pipetted into a beaker and the flask was returned to the bath. To the 20 cc. were added about 150 cc. of boiling water (to stop enzyme action), 8 drops of phenolphthalein and 30 cc. of neutralised formalin (1 part formalin: 2 parts H_2O) and the mixture was titrated with 0·1 *N* H_2SO_4. At intervals the flasks were shaken and a further 20 cc. of mixture were removed and treated in the same way.

When preliminary peptic digestion was employed the following method was adopted. 75 g. of minced meat were left with 75 cc. of water in an ice-chest overnight. The next day 75 cc. of 0·4 % HCl at 45° were added, together with 15 cc. of 10 % solution of pepsin scales. This mixture was left in a bath at 37° for 2 hours. The contents of the flasks were then neutralised with solid anhydrous Na_2CO_3. To the neutralised mixture 150 cc. of 0·8 % Na_2CO_3 at 37° and 20 cc. of 10 % pancreatic substance were added, and the contents were treated as described for tryptic digests.

When grilling meat a large piece of rump steak was divided into four parts. Two of these were grilled till bright red inside, one was over-cooked till brown throughout, and one was left raw. One of the two underdone steaks was allowed to cool, and was then re-heated till warmed through, but care was taken to stop at this point. The steak was thus re-heated but not over-cooked. The four samples of meat were allowed to cool, minced and treated as described for roast beef.

For experiments on boiled meat, a large piece of lean skirt steak freed from obvious connective tissue was minced. Portions of 75 g. were weighed into flasks together with 75 cc. of water. Some were left raw, some were immersed in boiling water for 10 minutes (when all the mince had turned brown) and some for 60 minutes. Half of the samples cooked for 10 minutes were allowed to cool and then re-boiled for a further 50 minutes. The flasks, after cooling, were made up to their original weight with water to keep the volume constant, and were then treated as described for roast beef.

Results.

The first set of results in each series is shown graphically in Fig. 1.

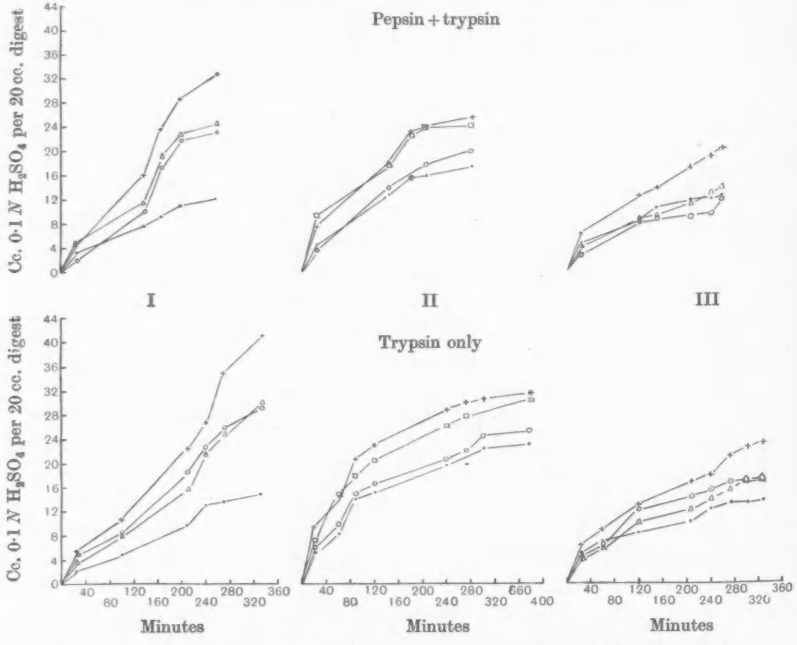

Fig. 1. I. Roast topside of beef. II. Grilled rump steak. III. Boiled skirt steak.
+—+— Cooked meat. ○—○— Over-cooked meat. △—△— Re-heated and over-cooked meat. □—□— Re-heated but *not* over-cooked meat. •—•— Raw meat.

Table I. *Roast meat (topside of beef).*

Cc. 0·1 *N* H_2SO_4 per 20 cc. digest after HCHO.

Mins.	Raw				Cooked				Over-cooked				Re-heated and over-cooked			
Pepsin + trypsin:																
30	3·7	4·4	3·8	4·0	4·2	4·8	5·7	4·7	2·2	3·6	3·0	4·4	4·5	2·5	3·4	4·7
135	7·8	8·0	7·5	8·3	16·0	10·7	14·6	16·5	10·0	—	10·8	12·2	11·2	10·8	11·4	9·8
165	8·9	9·5	8·1	10·1	23·5	20·1	22·7	24·0	17·1	13·6	15·2	16·9	19·0	13·9	16·1	17·1
195	11·1	11·6	11·5	11·8	28·2	22·9	27·5	28·4	21·7	18·2	20·6	21·6	23·4	15·2	19·8	20·7
255	12·0	12·7	12·6	12·9	32·4	25·7	29·3	31·1	23·4	19·0	22·1	22·9	24·2	16·4	20·5	23·1
Trypsin only:																
30	2·3	3·7	4·0	4·1	5·7	6·1	6·0	5·4	5·4	4·7	4·2	4·9	3·7	4·5	4·2	5·0
105	4·8	6·2	5·7	8·0	10·9	16·0	12·3	12·3	8·8	9·1	10·3	9·9	8·0	8·9	11·0	10·1
210	9·6	11·1	10·1	11·7	22·5	26·2	22·7	24·0	18·9	18·3	17·9	20·2	15·6	17·2	18·1	19·3
240	12·9	13·7	13·2	13·4	26·7	32·0	27·7	27·9	23·2	23·1	22·1	24·1	21·6	22·9	22·8	23·7
270	13·8	15·8	14·4	15·1	35·2	34·3	32·1	32·0	26·2	25·7	24·6	27·6	25·1	24·3	25·1	26·5
330	14·9	16·1	15·8	15·7	41·1	36·0	36·3	33·1	29·2	28·0	27·9	28·3	29·9	27·8	28·2	27·9

In the case of roast beef (Table I), contrary to expectations there was no difference in digestion rate if trypsin alone were used or if it were preceded by pepsin. Raw meat is seen to be far less readily digested *in vitro* than roast meat. The maximum rate of digestion was obtained when meat was roasted so that when cut after cooling it appeared a bright red colour with a juicy surface. Over-cooking till the cold joint on cutting appeared brown and dry (though quite edible), markedly slowed the digestive rate, and this slowing occurred to an equal extent whether the application of heat were continuous or conducted in two successive periods.

Table II. *Grilled meat (rump steak).*

Cc. of 0·1 *N* H_2SO_4 per 20 cc. digest after HCHO.

Mins.	Raw				Cooked				Over-cooked				Re-heated but *not* over-cooked			
Pepsin + trypsin:																
30	4·3	3·9	4·1	4·7	7·5	8·0	7·8	7·4	3·6	3·8	4·1	3·4	9·0	7·6	7·7	6·9
150	12·6	11·5	9·7	8·3	18·2	19·1	21·2	20·1	12·9	12·7	11·3	12·1	17·6	18·2	20·8	18·7
180	15·5	15·0	10·2	10·5	23·0	23·4	24·1	24·0	15·2	14·1	13·1	14·5	22·9	22·3	24·5	23·5
210	15·8	16·2	11·7	12·6	23·5	24·1	25·9	26·1	17·5	16·0	15·4	16·2	23·2	23·5	25·2	25·7
285	17·1	18·3	12·3	14·7	24·9	27·2	27·8	23·9	19·6	18·5	16·8	17·9	23·7	24·7	26·7	22·8
Trypsin only:																
30	5·3	4·0	4·7	5·0	9·2	8·0	8·5	8·2	5·7	5·3	4·0	4·6	7·1	8·0	7·9	8·2
60	8·2	7·8	8·5	7·9	14·4	14·7	15·1	14·6	10·2	12·2	10·2	11·8	14·8	14·4	15·2	14·3
90	13·9	11·9	12·1	12·6	20·4	18·8	19·2	20·0	14·7	14·2	13·7	13·9	17·8	18·2	17·5	17·1
120	15·1	12·0	14·4	13·9	22·8	20·8	21·7	23·1	16·6	15·8	15·9	16·0	20·6	20·9	21·0	20·2
240	19·5	18·3	18·8	17·8	28·4	28·1	27·8	28·6	20·9	19·7	20·0	20·4	26·1	27·1	26·2	27·4
270	19·7	20·2	20·0	19·2	29·6	28·9	28·4	29·2	22·2	20·6	22·7	21·9	27·9	28·0	28·4	28·7
300	22·6	21·0	20·9	21·5	30·7	30·2	29·6	29·8	24·4	22·0	23·8	23·7	30·4	28·4	29·1	29·6
375	22·7	22·3	22·3	21·9	31·6	31·1	30·9	31·7	25·3	23·0	24·0	24·9	30·7	30·3	30·0	31·0

With grilled meat again (Table II) no appreciable difference could be seen whether trypsin alone or pepsin and trypsin were employed, and in both series the digestion rate of raw meat was markedly slower than that of cooked meat. As with roast beef over-cooking retarded digestive action, but no appreciable difference could be discerned between the rate of digestion of lightly-grilled steak and that of a similar piece re-warmed but not over-cooked in the process. The slowing effect of re-cooking seen in the roast meat experiments

therefore appears to be due to the hardening of the protein rather than to the actual double application of heat with an intermediate cooling period.

Table III. *Boiled meat (skirt steak).*

Cc. 0·1*N* H_2SO_4 per 20 cc. digest after HCHO.

Mins.	Raw				Cooked				Over-cooked				Re-heated and over-cooked			
Pepsin + trypsin:																
30	4·4	2·3	3·9	4·7	6·0	6·8	6·5	5·9	2·9	3·1	2·6	3·2	4·2	3·9	4·1	4·0
120	8·3	7·9	8·0	8·5	12·3	12·0	13·2	12·7	8·1	8·4	8·5	8·7	8·2	8·0	7·9	8·5
150	10·6	10·8	9·7	11·1	13·6	14·1	14·4	13·8	8·3	10·2	11·2	10·0	8·7	10·2	10·5	10·3
210	11·7	12·0	11·6	12·3	17·0	16·9	17·0	16·5	8·8	10·9	12·1	11·4	11·3	11·8	11·4	12·0
240	11·8	12·2	11·9	12·6	18·7	17·6	18·2	18·4	9·2	11·0	11·2	11·7	12·7	12·1	12·4	11·9
270	12·2	13·0	12·4	12·9	20·0	20·5	19·7	20·4	11·9	12·1	13·6	12·3	13·5	12·7	13·1	12·7
Trypsin only:																
30	5·0	4·1	4·4	4·9	6·5	7·0	6·9	6·0	4·6	5·0	4·8	5·2	4·1	4·7	5·0	4·3
60	6·9	5·0	6·0	7·2	9·0	9·3	9·2	8·7	6·8	8·1	7·9	8·1	6·3	6·7	7·2	6·9
120	8·2	8·7	8·1	9·0	13·2	13·0	13·3	12·3	12·1	12·4	12·0	11·9	10·0	9·4	11·4	10·4
210	10·3	12·0	10·7	11·4	16·7	16·8	16·0	15·7	14·7	15·1	14·5	14·4	12·2	12·9	13·3	12·7
240	12·3	12·9	11·0	11·8	17·9	17·7	17·5	17·0	15·7	16·3	16·0	15·5	13·9	14·1	14·7	14·4
270	12·9	13·8	12·4	12·2	21·1	20·9	19·9	20·0	17·0	17·1	16·8	16·7	15·6	15·9	16·1	15·7
300	12·9	14·0	13·2	12·9	22·6	21·8	22·1	21·4	17·3	17·6	17·0	16·9	16·8	16·2	16·9	17·0
330	13·1	14·7	13·6	13·8	22·8	22·5	22·7	23·1	17·5	17·6	17·8	17·2	17·4	16·9	17·4	17·8

Similar results were obtained with boiled meat (Table III). The over-boiled mince in the set of experiments with pepsin and trypsin was digested more slowly than when trypsin alone was used. This is probably due to the fact that the boiled minced steak ran together in lumps when HCl was added and tended to do so throughout the experiment, whilst when Na_2CO_3 alone was used the meat remained in discrete particles. This aggregation was not seen with minces from grilled or roast meat.

Discussion.

From the above experiments it seems that, taking rapidity of hydrolysis as a criterion, meat when raw is in the least digestible condition. The most rapidly digested meat is that which is roasted or grilled until it is just red inside with a moist surface when cut. This point probably coincides with that at which the anti-enzymic properties of animal tissues are destroyed, whilst the hardening effect of heat on proteins has hardly come into play.

If the heating is carried beyond this point and the meat is definitely over-cooked, the rate of digestion is slowed but, unless the meat be actually carbonised, it even then appears to be more rapidly digested than raw meat. The least rapidly digested form of cooked meat found in these experiments is an over-boiled mince—the most rapidly, an underdone roast. It would therefore seem that for people with weak digestive powers or for young children the ideal form in which to administer animal protein is as underdone roast beef minced after cooking. This mince which comes from an ordinary household machine as a moist paste is very palatable.

The apparently very slow rate of digestion with boiled meat in these experiments may be due to the fine state of division of the meat before cooking,

and probably if a large joint, *e.g.* a leg of mutton, had been taken for boiling instead of minced steak, the differences between roast and boiled meats would not be so marked. Obviously it is difficult, if not impossible, to boil minced meat to a state comparable with that of an underdone roast joint. Experiments on these lines are in progress.

The decrease of digestibility in over-cooked meat appears to be due to hardening and drying of the protein since, if underdone roast or grilled meat is re-warmed but not over-cooked, its digestion rate is unaltered, but if it is re-heated until it is quite over-cooked the rate is slowed. This is in accordance with general opinion amongst cookery teachers who recommend the re-warming of meat but condemn re-cooking.

Summary.

Raw meat is digested *in vitro* much more slowly than cooked meat.

Over-cooked meat is very slowly digested as compared with underdone meat.

The maximum rate of digestion is obtained with underdone roast meat.

Re-warming underdone meat does not diminish its digestive rate. Re-heating with consequent over-cooking diminishes the rate of digestion.

The rate of digestion of meat (raw or cooked) is the same whether trypsin alone be used or pepsin followed by trypsin.

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CXCIII. DETERMINATION OF MALTOSE IN PLANT EXTRACTS BY MALTASE.

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Davis and Daish [1912–13] have shown that during the acid hydrolysis of a mixture of maltose and sucrose, appreciable quantities of the resulting laevulose and smaller quantities of dextrose are destroyed, thereby rendering an estimation of these sugars by acid hydrolysis erroneous. Under conditions recommended by Brown and Morris [1893] for hydrolysing maltose in presence of sucrose, about 30 % of the laevulose is destroyed. A double concentration of the acid, other conditions being the same, destroys nearly 90 % of laevulose and about 6 % of dextrose. Experiments on 1 % maltose solution showed that the hydrolysis of maltose (according to the method of Brown and Morris) had proceeded to the extent of 97 % at the end of 1 hour, increasing to 98 % at the end of 2 hours, but showing a lower value at the end of 3 hours, owing probably to the destruction of dextrose. Prolonged hydrolysis for 24 hours at a lower temperature (70°) accounted for only 94 % of maltose. These authors therefore resorted to fermentation methods, involving a long incubation period of 3–4 weeks, which from many points of view are disadvantageous. It therefore appeared desirable to use maltase for the estimation of maltose, as methods of preparing the enzyme in an active state had been worked out in great detail by Krieble *et al.* [1927].

Experimental.

The enzyme extract was prepared from finely ground brewer's yeast desiccated over phosphorus pentoxide by treating the material with 10 parts of a 5·8 % solution of sodium phosphate at 20° for 14 hours. The extract was centrifuged for 10 minutes and the clear centrifugate, after neutralisation to litmus with a 4 % KH_2PO_4 solution, was preserved at 0°.

A comparative study of the acid and enzymic hydrolysis of a solution of maltose (0·572 g. of pure maltose in 100 cc. of water) was made. Acid hydrolysis, under conditions recommended by Brown and Morris [1893], accounts for 97·5 % of maltose while enzymic hydrolysis accounts for 99·7 % of the sugar. The enzyme extract was used as the source of invertase for the hydrolysis of a 1 % solution of sucrose and an estimation of the invert sugar in

the hydrolysate accounted for 99·8 % of sucrose, thereby establishing the efficiency of the enzyme extract as a rich source of invertase as well.

Experiments were then conducted on a mixture containing 0·5 g. of maltose and 0·5 g. of sucrose in 100 cc. of water. A fresh enzyme extract as well as an old enzyme extract stored at 0° for 7 days were used for the hydrolysis experiments. The temperature and the quantity of the enzyme used for the hydrolysis were varied. In all cases, the hydrolysis was allowed to proceed for 36 hours after which the hydrolysate was clarified with 5 cc. of "dialysed iron," filtered, and the filtrate was made up to 50 cc., 20 cc. aliquots of which were employed for the estimation of the reducing sugar by Bertrand's method.

Another 20 cc. portion of the original sugar mixture was hydrolysed with 2 g. of citric acid for 10 minutes at 97° and after neutralisation with alkali to phenolphthalein, the reducing sugars were similarly determined.

Calculation. The reducing power of the citric acid hydrolysate corresponding to 5 cc. of the solution in mg. of copper is 77·1. The direct reducing power of 5 cc. of the solution in mg. of copper is 27·3. The difference between the two values is that due to invert sugar and, calculating the sucrose content from this, 99·6 % of the sucrose is accounted for.

If A is the reducing power of maltose in mg. of copper of a certain quantity of the solution, and Z and Y, the copper equivalents in mg. of the maltase hydrolysate and citric acid hydrolysate respectively, then

$$A = (Z - Y) \times \frac{212{\cdot}04}{147{\cdot}96}.$$

From this equation A can be calculated, and from the sugar tables, the corresponding quantity of maltose.

Table I.

			Enzyme experiment		
Hydrolysis	HCl 2 hrs.	HCl 3 hrs.	2·5 cc. 25°	5 cc. 25°	5 cc. 37°
Maltose found %	73·6	62·8	98·8	99·6	94·4

During acid hydrolysis the value of Z decreases largely due to the destruction of the laevulose portion of the invert sugar and also to the slight destruction of the dextrose, thereby reducing the value of maltose determined. It is clear from the above table that while acid hydrolysis is fatal in the estimation of sugar mixtures containing sucrose, the freshly prepared maltase extract is capable of hydrolysing quantitatively maltose and sucrose without destroying the products of hydrolysis. The sucrose in the mixture can be selectively hydrolysed by maltase-free invertase to obtain the value Y, while Z can be obtained by hydrolysing the mixture by the maltase extract which has been shown to be a rich source of invertase.

Estimation of added maltose in plant extracts by the enzyme method. Maltose (0·5291 g.) was added to 50 cc. of tissue fluid from healthy sandal leaves, after clarification with "dialysed iron" to eliminate tannins, proteins etc. 10 cc. portions of this solution containing the added maltose were hydrolysed with

3 cc. of the enzyme extract for 36 hours. Another 10 cc. portion of the clarified tissue fluid without added maltose was hydrolysed under identical conditions and sugar was estimated in 20 cc. of the total solution.

The difference between the above two sets of results gives the reducing power of the added maltose after hydrolysis and in this experiment 96·22 % of the added maltose was found.

Summary.

A reliable method for the estimation of maltose in mixtures of sugars occurring in plant extracts and tissue fluids has been described wherein an enzyme extract rich in both maltase and invertase has been used as the hydrolysing agent. The method is selective in its action and completely eliminates the errors inherent in the method of acid hydrolysis.

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CXCIV. THE CORRELATION OF THE OXIDATION OF CERTAIN PHENOLS AND OF DIMETHYL-*p*-PHENYLENEDIAMINE BY BACTERIAL SUSPENSIONS.

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Gordon and McLeod [1928] have shown that the colonies of most bacteria which grow well only in the presence of oxygen, when treated with a 1 % solution of dimethyl-*p*-phenylenediamine hydrochloride, assume a reddish tint which progressively darkens to black. This they attributed to the presence in such bacteria of a direct oxidase system the relative thermolability of which was shown when the bacteria were heated to 88° for 1 hour. Nishibe [1926], following Schultze's [1910] use of a mixture of dimethyl-*p*-phenylenediamine with *α*-naphthol in the study of the structure of the anthrax bacillus, used this same indophenol reagent in an investigation similar to that of Gordon and McLeod and obtained similar results.

During the course of some routine work a young culture of *B. pyocyaneus* was treated with the dimethyl-*p*-phenylenediamine reagent. Rapid pigmentation commenced but almost immediately a rapid fading process was superimposed and in a few minutes only a fringe of colour round the periphery of the culture plaque was discernible. The culture was again treated with the reagent and with a similar result except that a larger portion of the culture became irreversibly pigmented. This phenomenon was attributed to bacterial reduction, and, since Vernon [1911] and Batelli and Stern [1912] have shown that the oxidation of this reagent in the presence of *α*-naphthol may be greatly inhibited by reducing systems, it seemed possible that this "direct oxidase" reaction might be missed by the technique of Gordon and McLeod in bacterial systems where it was present but masked by reducing systems. This possibility has been indicated by Ellingworth, McLeod and Gordon [1929]. It was also felt that the argument of Gordon and McLeod would be strengthened if it could be shown that those organisms which give the dimethyl-*p*-phenylenediamine reaction could also catalyse the oxidation of certain phenols to substances which could be chemically isolated and identified as products of oxidation. Happold and Raper [1925 and unpublished results] have shown that tyrosinase in the presence of atmospheric oxygen and certain phenols which can be oxidised to form *ortho*-quinones causes the deamination and

decarboxylation of amino-acids, a reaction first reported by Chodat and Schweizer [1913] but attributed by them to the direct action of the enzyme on the amino-acid in the presence of oxygen. The aldehyde which contains one carbon atom less than the parent acid, ammonia and carbon dioxide are the final products of the reaction. They also showed that phenols which do not form *ortho*-quinones by oxidation are devoid of such action; *p*-benzoquinone can exert no such effect on amino-acids in the presence of oxygen but synthetic *o*-benzoquinone will cause deamination and decarboxylation of the amino-acid without the addition of the enzyme. Attempts to isolate *o*-benzoquinone from the system tyrosinase-catechol-oxygen were not made because of the instability of the quinone, but Pugh and Raper [1927] successfully proved that *o*-benzoquinone and homoquinone are products of the aerobic action of tyrosinase on phenol or catechol and *p*-cresol respectively. This they accomplished by isolating the dianilino-derivatives of the quinones and by establishing the chemical identity of the isolated derivatives.

In the present investigation the technique of these authors has been followed in most essentials in testing the ability of bacterial suspensions to catalyse the oxidation of phenols. Well washed, heavy bacterial suspensions buffered in phosphate at p_H 6·5 have been used throughout. The colour changes produced when solutions of catechol, guaiacol, orcinol, phenol, *p*-cresol and tyrosine are treated with washed bacterial suspensions have been noticed and compared with the results obtained by following the technique of Gordon and McLeod. None of the organisms tested has been capable of causing colour changes in solutions of phenol, *p*-cresol or tyrosine with the exception of a strain of *Vibrio tyrosinatica* obtained through the courtesy of Prof. Kluyver of Delft, but changes have been noticed with solutions of catechol, guaiacol and orcinol. Increased deamination of amino-acids in the presence of catechol appears to be restricted to suspensions of those organisms which produce colour changes in catechol and the production of *o*-benzoquinone in such systems has been demonstrated by the isolation of the dianilinoquinone from the system aniline, catechol and oxygen with suspensions of cholera and an anthracoid respectively. Guaiacol was oxidised in the presence of oxygen to a maroon-coloured compound by the anthracoids studied, but no deamination took place in the system anthracoid-guaiacol-alanine. A lemon-yellow colour is also produced with orcinol by certain organisms. The chemical nature of the coloured compounds produced is not known but there is evidence that compounds containing the *ortho*-quinone grouping are not produced in the oxidation.

Colour changes produced with phenolic substances in the presence of oxygen by bacterial suspensions. Cultures of organisms were made on agar plates or, in the case of delicate bacteria, on blood-agar plates. After 24 hours the cultures were suspended in sterile saline and the bacteria were then separated from the saline by centrifuging. The deposited bacteria were suspended in phosphate buffer at p_H 6·5 so that a suspension of milk-like opacity was

obtained and this suspension was divided and one-half was heated for 30 minutes in a boiling water-bath; the boiled and unheated suspensions were divided into 2 cc. portions and 1 cc. of a 1 % solution of phenol, *p*-cresol, catechol, or orcinol, or 1 cc. of a saturated solution of tyrosine or guaiacol, was added in duplicate to the two suspensions. The action of dimethyl-*p*-phenylenediamine hydrochloride on a plate culture of the organism was also tested for purposes of comparison. Colour changes were observed during the first 3 hours and after 18 hours and the latter results have been recorded. A further control was made by adding 2 cc. of buffer to 1 cc. of the solution containing the phenol.

Table I.

Suspension tested	Substrate: Dimethyl-*p*-phenylene-diamine	Catechol	Guaiacol	Orcinol	*p*-Cresol, phenol and tyrosine
Anthracoid	+ + +	+ + +	+ +	+ +	−
Do. boiled	.	+ +	−	Trace	−
B. subtilis	+ +	+ + +	+ +	.	−
Do. boiled	.	+ +	−	.	−
Cholera	+ + +	+ +	−	−	−
Do. boiled	.	−	−	−	−
Gonococcus	+ + +	+ +	−	+	−
Do. boiled	.	−	−	−	−
Meningococcus	+ + +	+ +	−	−	−
Do. boiled	.	−	−	−	−
Gram-negative diplococcus (throat)	+ + +	+ +	−	.	−
Do. boiled	.	−	−	.	−
B. pyocyaneus	+ ±	+ ±	−	−	−
Do. boiled	.	−	−	−	−
B. prodigiosus	+ ±	+	−	+ +	−
Do. boiled	.	−	−	−	−
Sarcina	+	+ +	−	+	−
Do. boiled	.	−	−	Trace	−
Staph. albus	−	+	−	+	−
Do. boiled	.	−	−	−	−
Vibrio tyrosinatica	+ +	+ + +	−	.	+ + +
Do. boiled	.	−	−	.	−

Negative results were obtained with the following, Streptococcus isolated from case of puerperal sepsis, *B. pfeiffer*, three strains of Sarcina, *B. welchii*, *B. sporogenes*, *C. diphtheriae*, *B. coli*, *B. aertryck* "Mutton" and "Newport."

The next stage in the investigation was to show that those organisms which gave the colour changes with the dimethyl-*p*-phenylenediamine and with the catechol could also cause deamination of amino-acids in the presence of the latter reagent with the liberation of ammonia. Previously this had been studied by following not only the liberation of ammonia in the system enzyme-catechol-amino-acid-buffer, but also the decrease in the amino-nitrogen content of the system as oxidation of the catechol to *o*-benzoquinone proceeded. In the present investigation the liberation of ammonia has been taken as sole evidence of deamination. Four test aeration bottles

were prepared as follows: (1) 30 cc. of phosphate buffer (p_H 6·5) 0·2 g. of alanine, 0·1 g. of catechol and 10 cc. of a washed suspension of the organism to be studied; (2) differed only from (1) in that the bacterial suspension had been immersed in a boiling water-bath for 30 minutes; (3) as (1) except that no catechol was added; (4) differed from (1) since no bacterial suspension had been added. Chloroform was now added to all four bottles to depress any possible bacterial growth in system (3). Ammonia-free air was aspirated for 24 hours through the four bottles and was then caused to pass through four wash-bottles which contained 25 cc. of $N/10$ H_2SO_4. After this period of aeration a few drops of capryl alcohol and 2–3 g. of sodium hydroxide were added to each reaction bottle and any ammonia which was liberated was rapidly aspirated into the bottles containing the standard acid. The ammonia could then be estimated. The first apparent difficulty lay in the observed fact that, though non-enzymic oxidation of catechol could not be observed at the reaction (p_H 6·5) at which the oxidations were being carried out, when the alkali was added for liberation and estimation of the ammonia the oxidation of catechol could proceed rapidly without the intervention of any enzyme system and presumably by a similar type of oxidation to that found in the enzyme system. Thus if the control bottle (4), containing no bacterial suspension, was taken and was aerated for 24 hours, there would be practically no trace of pigment, and the production of *o*-benzoquinone as stated by Pugh and Raper [1927] could not be demonstrated at this stage. If now the 2–3 g. of NaOH were added, a rapid pigmentation of the mixture proceeded accompanied by the deamination of the amino-acid. The rate of this non-enzymic deamination has been measured; the average ammonia liberated in such a system during the first 40 minutes of rapid aspiration was so slight that it was within the limit of experimental error. After 80 minutes it represented on an average the equivalent of 0·14 cc. of $N/10$ NaOH, after 160 minutes, 0·31 cc. and after the whole system had been left standing overnight and then aspirated rapidly for a further period of 40 minutes in the morning the total ammonia which had been liberated was equivalent to 1·21 cc. of $N/10$ NaOH. In view of these findings the period of rapid aspiration subsequent to the addition of the NaOH was limited to 60 minutes, a period which had been shown to be adequate for the liberation and estimation of such amounts of ammonia as were likely to be present, and in which the ammonia formed and liberated by the non-enzymic oxidation of the catechol averaged approximately 0·15 mg. NH_3.

Table II, column 1 represents the difference between the average estimated ammonia content of the system (amino-acid-buffer-bacterial suspension-catechol) or of bottle (1) and that of bottle (4) which represents the ammonia liberated by non-enzymic action (0·15 mg.). Column 2 gives the same difference between bottle (2) and bottle (4) and this result shows us whether a bacterial suspension possesses any heat-stable catalyst for the oxidation of catechol. Column 3, which records the values obtained for bottle (3), affords

a combined measure of any volatile basic substances present in the bacterial suspension and liberated under the conditions of the experiment, and of any ammonia produced by normal deamination of the alanine by the bacterial suspension.

Table II.

Mean values of ammonia content expressed in mg.

Organism used	System 1–system 4	System 2–system 4	System 3
Anthracoid	1·05	0·38	0·17
B. subtilis	0·81	0·05	0·25
Cholera	0·72	0·08	0·25
Gonococcus	0·66	−0·04	0·10
B. pyocyaneus	0·69	0·20	0·20
B. prodigiosus	0·86	0·26	0·37
V. tyrosinatica	0·89	0·22	0·25
Streptococcus	0·19	0·09	0·14
B. aertryck "Newport"	0·06	0·10	0·19
Staphylococcus	0·07	Nil	0·29

These results are in general the mean of three experiments. The difference in value between the results recorded in columns 1 and 3 for any one organism is a measure of the deamination caused by the enzymic oxidation of the catechol by the organism concerned and it is at once apparent that all those organisms which give the dimethyl-*p*-phenylenediamine reaction catalyse the oxidation of catechol. The results given in column 2 on the other hand indicate that with the possible exception of the anthracoid all other organisms lose this catalytic effect after subjection to a temperature of 100° for 30 minutes.

Various other phenols were now substituted for catechol *viz.* phenol, *p*-cresol and guaiacol and since these substances were not autoxidised the control bottle (4) could be withdrawn. The results can be briefly summarised as follows: of all the organisms tested none could cause deamination of amino-acids in the presence of the monohydric phenols, except the tyrosinase-containing *V. tyrosinatica* and though the two spore-bearing aerobes used catalysed an oxidative pigmentation of the guaiacol, neither they nor any of the other organisms tested could catalyse oxidative deamination of amino-acids in the presence of guaiacol. The following results indicate these facts. *B. subtilis* is taken as a type for the rest of the organisms which do not catalyse the oxidation of monohydric phenols and *p*-cresol as the representative of the two monohydric phenols used.

Table III.

Mean value of ammonia content expressed in mg.

Organism used	Suspension-*p*-cresol amino-acid	Heated suspension-*p*-cresol-amino acid	Suspension amino-acid
B. subtilis	0·09	0·09	Nil
V. tyrosinatica	1·05	0·40	0·51

The high ammonia value of the heated *V. tyrosinatica* suspension can be understood when one realises that the suspension is heated at a slightly acid reaction, so that there is not the same tendency to drive off ammonia and volatile bases by heating as there would be if the suspensions were made in

a neutral liquid. Since cultures of the organism are also of a mucilaginous consistency, satisfactory purification by washing is extremely difficult. The ammonia value in the absence of *p*-cresol is consequently high.

Action of washed bacterial suspensions on catechol in the presence of aniline. 2 g. of catechol, 4 cc. of aniline and the washed suspension prepared from 10 plate cultures of (1) the anthracoid and (2) *V. cholera* respectively were made up to 1 litre with Clark's buffer mixture (p_H 6·5) and the whole was aerated for 48 hours as recommended by Pugh and Raper [1927]. The reddish precipitate which separated in each of the two preparations was filtered off and washed alternately with 1 % HCl and distilled water so as to remove traces of aniline; it was then dried *in vacuo*. The dried filter papers were then extracted with ether in a Soxhlet apparatus and the ethereal solution was extracted with 0·02*N* NaOH; the aqueous layer was separated and acidified with 2 % HCl and the precipitate which formed was again filtered off and dried as before. The precipitate was finally extracted with acetone and was crystallised from this solvent. The crystals which were obtained in both experiments were bright reddish-coloured needles which gave all the reactions characteristic of the anilino-*o*-benzoquinone prepared by Pugh and Raper. The yield with the anthracoid was approximately 1·4 g. of the crystals having M.P. 193·5°, N, 9·61 %. The yield with cholera was approximately 1·3 g. of crystals, having M.P. 193·0°, N, 9·57 %. The highest M.P. found by Pugh and Raper was 193·5°, and $C_{18}H_{14}O_2N_2$ requires N, 9·66 %. Efforts to obtain anilino-quinones from control experiments using boiled bacterial suspensions were unsuccessful, though some pigmentation occurred.

The oxidation of catechol in the presence of aniline at alkaline reactions. The observation that catechol was oxidised at alkaline reactions without the intervention of any catalyst and that the oxidation proceeded by such channels that amino-acids if present were concurrently deaminated led the author to investigate whether or not *o*-benzoquinone was produced as an intermediate product. The investigation has been extremely difficult since there appeared to be a marked tendency to tar-formation. Aeration experiments were carried out on the same quantities of catechol and aniline as before, but the solutions were buffered at p_H 9·0 and no bacterial suspensions were added. The aeration was carried out for 48 hours. A first experiment gave reddish-coloured crystals whose M.P. was low and indeterminate; the amount was too small for further experimentation. A second experiment revealed two distinct types of crystals separating from an acetone solution of the products of the reaction, (1) typical reddish-coloured needles whose appearance was identical with that of anilino-*o*-benzoquinone and (2) orange-yellow coloured hexagonal plates. Both types of crystals came down together though the latter predominated. The yellow crystals have M.P. $> 260°$ and differ from anilino-*o*-benzoquinone in that they are not readily soluble in *N*/50 sodium hydroxide.

SUMMARY.

The capacity of bacterial suspensions to catalyse the oxidation of catechol, guaiacol, orcinol, phenol, *p*-cresol and tyrosine has been studied and it has been shown that the oxidation of catechol with the formation of *ortho*-benzoquinone is only catalysed by those organisms which give the dimethyl-*p*-phenylenediamine reaction as applied by Gordon and McLeod. An apparent discrepancy exists with the strain of Staphylococcus used, since suspensions of the organism gave a typical though weak colour change with catechol and then failed to give evidence of deamination in the system bacterial suspension-alanine-catechol. Catechol which does not appear to be appreciably autoxidised at slightly acid reactions is markedly oxidised at p_H 9·0 and when the oxidation is carried out in the presence of aniline two distinct crystalline substances, one of which appears to be the anilino-*o*-quinone, can be isolated from the system.

The anthracoids studied differ from the other oxidase-containing bacteria in catalysing the oxidation of guaiacol to a maroon-coloured compound of unknown type, whilst *V. tyrosinatica* catalyses the oxidation of phenol and *p*-cresol, phenols which do not appear to suffer autoxidation.

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CXCV. THE ALCOHOL-SOLUBILITY OF THE ANTI-DERMATITIS, MORE HEAT-STABLE VITAMIN B_2 CONSTITUENT OF THE VITAMIN B COMPLEX.

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The relative insolubility in alcoholic solutions of 70 % strength upwards of vitamin B_2 as compared with the antineuritic vitamin B_1, has been shown by several independent workers using materials derived from different sources [Goldberger *et al.*, 1926; Sherman and Sandels, 1929; Chick and Roscoe, 1929; Narayanan and Drummond, 1930]. Osborne and Wakeman [1919] used alcohol fractionation in the preparation of their "vitamin B" concentrate. Alcohol was added to a watery yeast extract, obtained by boiling brewer's yeast in dilute acetic acid, until the concentration in the mixture was 52 % (by weight), and the precipitate was removed. The additional precipitate formed on raising the concentration of alcohol in the filtrate to 79 % was found to contain "most of the water-soluble vitamin originally present in the yeast." It is probable that in the vitamin tests of these authors the growth of the experimental rats was limited by the amount of vitamin B_1 present, this factor being soluble in 70 % alcohol, but there can be little doubt that vitamin B_2 was abundantly present in the precipitate.

Chick and Roscoe [1929] added alcohol to a concentrated watery extract of brewer's yeast until the mixture contained 83–93 % alcohol by weight, and although vitamin B_2 could not be detected in the alcoholic solutions which were rich in vitamin B_1, the vitamin B_2 potency of the insoluble material was nevertheless found to be less than that of the original yeast extract. This result suggested that vitamin B_2 had suffered some destruction from the strong alcoholic environment.

Sherman and Sandels [1929] noticed a similar diminution in the total vitamin B_2 value when air-dried baker's yeast was extracted with 60–80 % alcohol. No loss, however, was detected when 95 % alcohol was used, the insoluble material in this case possessing potency equal to that of the original yeast. Chick and Roscoe also detected no loss when their yeast extract was evaporated to complete dryness and then treated with 96 % alcohol. Fixsen (unpublished experiments) using dried egg-white obtained a similar result with 98 % or absolute alcohol. From these experiments it would appear that

vitamin B_2 was sensitive to alcohols of strength ranging from about 60 to 95 %, but could resist stronger solutions.

Narayanan and Drummond [1930] have recently published results differing somewhat from the above. They employed alcoholic fractionation to prepare a strong vitamin B_2 concentrate from brewer's yeast. Starting from the vitamin B_2-containing fraction, obtained by decomposition of the precipitate formed in the yeast extract with lead acetate, alcohol was added to 50 % strength and the precipitate removed. To the filtrate "which contained about 88 % of the activity of the original fraction" alcohol was further added to approximately 70 %. The "slimy, dark brown, precipitate" was found to contain most of the original vitamin B_2 activity, and the organic matter contained in the daily dose required to maintain satisfactory growth in young rats was as low as 6 mg.

The value of such a vitamin B_2 concentrate for the further study of the vitamin B complex is obvious, and we have attempted to repeat the work of Narayanan and Drummond. Our efforts, described in the following paragraphs, have, however, met with little success.

We used the solution (Fraction C) obtained by decomposition of the precipitate formed (at p_H 7·5) when basic lead acetate is added to the extract obtained by boiling washed brewer's yeast in 0·01 % acetic acid solution [Chick and Roscoe, 1929]. The precipitate was decomposed and lead removed by H_2S, after acidification with sulphuric acid, and the filtrate concentrated on a water-bath until the equivalent of 745 g. yeast (dry weight) was contained in 3050 cc. (Fraction C), the p_H being 1·5.

The vitamin B_2 content of this and the succeeding fractions was estimated by the method previously described [Chick and Roscoe, 1928]. Before testing, the solution was brought to *ca.* p_H 3·0 and most of the sulphate present removed by addition of baryta and barium chloride. The final product was evaporated until 1 cc. contained the equivalent of 0·35 g. yeast (Control solution I). A daily dose of 1 cc. of this product maintained an average weekly increase in the body weight of young rats of 15·5 g., *i.e.* contained adequate vitamin B_2 for optimal growth ("normal" standard = average weekly increase of 10–12 g.).

Exp. 1. *Fractionation with alcohol at* p_H *1·5.* To 500 cc. of Fraction C (≡ 122 g. yeast) 96 % alcohol (by weight) was added until the concentration in the mixture was 56 %. A small precipitate (Precipitate II, dry weight 1·3 g.) separated, which was found to be inactive in doses equivalent to 6 g. yeast.

One-half of the filtrate (≡ 61 g. yeast) was reserved for testing. The alcohol was driven off by distillation under reduced pressure and the solution concentrated further on a water-bath. Baryta was added to remove sulphate and to bring the solution to *ca.* p_H 3·0. This fraction (Filtrate II) possessed about half the vitamin B_2 potency of the Fraction C to which it corresponded. The daily dose required for normal growth was equivalent to 0·35–0·7 g. yeast (average weekly increase in weight 10–12 g.).

To the other half of Filtrate II (≡ 61 g. yeast) 96 % alcohol was added till the concentration of the mixture was 70 %. A very small precipitate was formed on neutralising the solution; this proved inactive in doses equivalent to 1·4 g. yeast (Precipitate III). The filtrate (Filtrate III), tested after preliminary treatment similar to that of Filtrate II, was also found to be inactive in doses equivalent to 1·4 g. yeast. A combined dose of Precipitate III and Filtrate III, each equivalent to 1·4 g. yeast, was equally ineffective.

Exp. 2. *Fractionation with alcohol at* p_H *3·2.* An experiment similar to Exp. 1 was carried out with 500 cc. of Fraction C (≡ 122 g. yeast) after neutralisation to p_H 3·2. The precipitate formed with 56 % alcohol (Precipitate IV) contained more sulphate and was larger than Precipitate II in Exp. 1. Traces only of activity, however, were detected in doses equivalent to 1·5 g. yeast. The filtrate (Filtrate IV) was equally inactive.

When Filtrate IV was subsequently fractionated with 70 % alcohol the small precipitate thrown down on neutralisation (Precipitate V), the filtrate (Filtrate V) and a combination of both, were all found to be inactive. The doses given were the equivalents of 1·5–2·2 g. yeast.

The results of these two experiments may be summarised thus. Precipitation with 56 % alcohol at p_H 1·5 (Exp. 1) caused the disappearance of about one-half of the vitamin B_2 originally present, while subsequent treatment with 70 % alcohol caused destruction or inactivation of the remainder. With less acid material, p_H 3·2 (Exp. 2), inactivation appeared to take place with 56 % alcohol.

Experiments were also made with a vitamin B_2 preparation prepared from egg-white [Chick, Copping and Roscoe, 1930]. When fractionation was attempted with 50 % alcohol at p_H 4·0, destruction of at least half of the vitamin occurred; with 70 % alcohol destruction appeared to be complete. An attempt to make a vitamin B_2 preparation by boiling acidified fresh egg-white with 50 % alcohol instead of water also proved unsuccessful.

The discrepancy between these results and those of Narayanan and Drummond (and possibly those of Osborne and Wakeman also) may be due to differences in the reaction of the solutions employed. The vitamin B_2 preparation fractionated by them was previously neutralised while those here described were acid, p_H 1·5 and 3·2 respectively. In the present study, however, vitamin B_2 appeared to be more stable in the more acid solution.

Such discrepancies are by no means uncommon in the scanty literature which exists concerning the properties of vitamin B_2. For example, this vitamin was found by Levene [1928] to be sensitive to the action of nitrous acid; but completely resistant by Chick and Roscoe [1929] and Narayanan and Drummond [1930]. Chick and Roscoe [1930] and Chick and Copping [1930] have found vitamin B_2 to be destroyed by action of alkalis (p_H 9–10) at high temperatures 120–125°; Reader [1929] and Narayanan and Drummond [1930], on the other hand, have found it to be resistant.

Vitamin B_2 (in common with the additional constituents of the B group

of vitamins recently described by Williams and Waterman [1927, 1928] and Reader [1929, 1930]) is probably not a well-defined or stable chemical entity. Its solubility and precipitation limits are irregular and uncertain and are greatly influenced by small variations in the conditions under which they are studied. These conditions include the dilution and hydrogen ion concentration of the solution and the presence and concentration of salts and other substances. There must also be other disturbing substances which are at present unrecognised. In this laboratory it has often proved impossible to repeat a given observation under apparently similar conditions. Much more investigation is needed to eliminate these difficulties and, in the meantime, the necessity for including exact details of procedure in all published work is obvious.

A further complication arises from the fact that the usual method for estimation of vitamin B_2 consists in a growth test with young rats and therefore is very liable to erroneous interpretation unless strictly controlled. A more satisfactory criterion would be the cure of the dermatitis which occurs in rats on diets deprived of this vitamin, but the skin lesions are irregular and slow of development (8–30 weeks) so that such tests are impracticable on a large scale.

SUMMARY.

When alcohol to 56 % was added to a solution of vitamin B_2 at p_H 1·5, prepared from brewer's yeast, the precipitate was inactive and the filtrate possessed only one-half the potency of the original material. After further addition of alcohol to 70 % the solution was completely inactive. When the original solution was less acid (p_H 3·2) total inactivation occurred with 56 % alcohol.

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CXCVI. EGG-WHITE AS A SOURCE OF THE ANTIDERMATITIS VITAMIN B_2.

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It has already been recorded that the fresh egg-white of the hen's egg contains vitamin B_2 unaccompanied by the antineuritic vitamin B_1 [Chick and Roscoe, 1929, 1]. In this property egg-white stands alone amongst the foodstuffs hitherto examined, for in all others in which vitamin B_2 has been found, vitamin B_1 has also been present, although the relative amounts of the two have shown a wide variation [Aykroyd and Roscoe, 1929; Roscoe, 1930].

This unusual property makes egg-white a valuable material for use in the investigation of the various members of the B group of vitamins. It has, therefore, seemed worth while to publish a short account of the evidence upon which the above statement is based.

Vitamin B_1 deficiency of egg-white.

If young rats immediately after weaning receive an artificial diet deprived of vitamin B_1, but otherwise complete and containing egg-white as source of protein and of vitamin B_2 [see Chick and Copping, 1930, 3, p. 1765, Diet II], growth takes place for 2–3 weeks, after which appetite fails and a decline in weight sets in. If this is not checked death will often follow and will be preceded by the paralysis characteristic of vitamin B_1 deficiency, which is promptly cured if vitamin B_1 is given. If, at the point where weight has become stationary or has begun to decline, vitamin B_1 is given as a daily dose of some foodstuff containing it, or as Peters's antineuritic concentrate, growth is immediately restored and the gain in weight will be in relation to the amount of vitamin B_1 administered. These facts have been applied in a method for estimation of vitamin B_1 in foodstuffs [Chick and Roscoe, 1929, 1].

Vitamin B_2 content of egg-white.

The content of vitamin B_2 can be estimated by measuring the minimal daily dose required to restore growth which has ceased in young rats after short periods, 1–2 weeks, on a synthetic diet containing vitamin B_1 and lacking only vitamin B_2 [Chick and Roscoe, 1928]. It has been found that a daily dose of 2·5–5·0 g. of fresh, cooked, egg-white (dry weight 0·3–0·6 g.) or of an

extract (see below) equivalent to 5–10 g. will restore "normal" growth (average weekly increase in weight of 10–12 g.). This result places egg-white among the foodstuffs richer in vitamin B_2, being of about equal value with milk and meat (reckoned on the dry weight), though much inferior to liver and yeast [Aykroyd and Roscoe, 1929].

Additional, more conclusive, evidence that vitamin B_2 is abundantly present in egg-white has been obtained from the cures which take place when the fresh material, or concentrates prepared therefrom, are administered to rats suffering from the characteristic skin lesions which develop in young rats after several weeks of vitamin B_2 deprivation.

A full description of these lesions is contained in a recent paper [Chick and Copping, 1930, 1; see also Chick and Roscoe, 1928]. The more "florid" dermatitis of ears and paws (see rats 513 and 529, Table I) has been rarely seen since the caseinogen in the basal diet has been carefully purified and it seems possible that its development is encouraged if the diet is not entirely free from traces of vitamin B_2 [see also Leader, 1930].

The details of some typical cures are set out in Table I. For these tests vitamin B_2 concentrates were prepared from egg-white after removal of the coagulable proteins as follows.

Fresh egg-white is diluted with 3·5 times its weight of distilled water and sulphuric acid added (*ca.* 4 cc. to 1000 g. egg-white) till the p_H is 4·2 (bromocresol green). The mixture is raised to the boil and boiled for about 5 minutes, care being taken to keep the mixture well stirred owing to its tendency to froth. The coagulum is filtered with slight suction and pressed in a hand-press, the press liquor being added to the filtrate. The press cake is weighed, and the quantity of adherent filtrate calculated on the assumption (which has been verified) that egg-white contains 10 % coagulable protein. Allowance for this loss is made in reckoning the amount of egg-white corresponding to the collected filtrate. This is evaporated in flat dishes under a fan in a hot room at 37° and afterwards on a water-bath at 100° till 1 cc. contains the equivalent of about 4–5 g. egg-white. The sulphates present are removed by addition of barium chloride solution, after making a preliminary trial with an aliquot portion to indicate the required amount. The removal is made slightly incomplete to avoid any risk of administering traces of barium to the experimental animals. The concentration of the filtrate is then continued until 1 cc. contains the equivalent of 5 g. egg-white and the p_H is adjusted to about 3·0 for storage.

A similar concentrate can be prepared with acetic in place of sulphuric acid. If the egg-white is diluted 4–5 times and acetic acid added to a concentration of 0·3 %, the p_H will be found optimal for precipitation of the protein. Separation and filtration of the coagulum is usually easier than in the sulphuric acid preparation, but the large amount of acetate in the final product is a drawback, rendering it unpalatable and likely to cause diarrhoea. Preparations made with sulphuric acid, on the other hand, are usually well taken and, if not entirely acceptable, can be made so by adding a few crystals of sugar to the daily dose.

Rats 400, 403 and 405 (Table I) had not suffered vitamin B_2 deficiency long enough for the development of skin lesions but show the effect of cooked fresh egg-white and of the purified concentrate in restoring growth after 7 weeks' deprivation. The restoration of growth, however, was not permanent and the rate fell off after a few weeks, irrespective of the size of the daily ration. This failure, which seems to vary in degree according to the duration of the previous period of deprivation, we believe is not to be attributed to

Table I. *Vitamin B_2 content of egg-white products.*

Restoration of growth and cure of dermatitis in young rats previously maintained on a diet (P2L) deprived of vitamin B_2; vitamin B_1 provided as a daily dose 0·1 cc.≡0·6 g. yeast of Peters's antineuritic concentrate [Peters, 1924; Kinnersley and Peters, 1925, 1927].

Material	Dose, expressed as equivalent of fresh egg-white g.	Rat No. and sex	Time previously on "$-B_2$" diet Weeks	Symptoms and general condition at beginning of cure	Duration of treatment Weeks	Body weight: At beginning of cure g.	Body weight: Total increase during treatment g.	Body weight: Average weekly increase g.	Result of treatment
Fresh egg-white	2·5	403 ♀	7	*No skin symptoms.* Body weight stationary	8	51	50	6·5	Growth good at first, but rate decreasing towards end of the period
,,	5·0	400 ♂	7	,, ,,	10	59	73	7	,, ,,
Egg-white 3 concentrate (acetic acid)	5	405 ♀	7	,, ,,	8	46	66	8·5	Growth good at first, but weight stationary during last 2 weeks
,,	5	387 ♀	16	*Skin symptoms moderate.* Eyes sunken "spectacled." Fur very poor, stringy in places, epidermis peeling off nose and mouth, abdomen stained	4	38	30	7·5	*Good cure.* Marked improvement in 7 days, eyes better, skin healthy and new fur beginning to grow. In 3 weeks coat good and eyes nearly normal. Increase in weight subnormal and not improved by doubling the dose
Egg-white 3 concentrate (acetic acid)	5	392 ♂	13	*No definite dermatitis.* Both eyes closed, eyelids stuck together, general condition miserable	5	47	49	10	*Cure.* After 7 days epidermis on face desquamating; after 3 weeks skin and fur normal, eyes nearly normal
Egg-white 11 concentrate (acetic acid)	5 later 10	513 ♀	22	*Skin symptoms severe.* Eyes sunken "spectacled." Skin of ears "papery." Fur "moist," "stringy," raw place under chin. Abdomen stained. General condition miserable	2 3	52	20 33	10·5	*Rapid cure.* Marked improvement in 5 days, eyes less sunken, skin beginning to look healthy, raw patch healed, appetite improved. In 4 weeks skin normal

Egg-white 11 concentrate (acetic acid)	2·5 later 10	529 ♀	21	*Skin symptoms severe.* Eyes sunken, "spectacled," right almost closed. Fur poor, raw patches of skin on fore limbs and on right chest, marked, "florid" dermatitis on fore paws	2 3	47	13 37	10	*Slow cure.* Definite improvement in 7 days, slower than with rat 513. Raw patches not healed in 12 days. Skin healed and coat normal after 3 weeks on increased dose
Egg-white 18 concentrate (sulphuric acid)	10	663 ♂	30	*Skin symptoms severe.* Eyes sunken and secreting. Coat very bad, moist and stringy; dry scurfy patches on haunches. Raw place slowly developed during 6 weeks on right fore limb, extending from neck to paw. General condition bad, dropped penis	3	99	29	10	*Slow cure.* General condition deteriorated during first few days with development of helplessness in hind limbs and spasticity of muscles; marked improvement in this and skin condition after 9 days. After 2 weeks raw place on right fore limb almost completely healed. In 3 weeks skin soft and healthy, new fur growing. Penis still dropped
Egg-white 18 concentrate (sulphuric acid)	10	710 ♂	19	*Skin symptoms moderate.* Eyes sunken and secreting. Nose and paws blood stained. Fur poor, nearly bald on head and neck, dry scurfy patches on haunches. General condition poor, dropped penis	3	56	39	13	*Rapid cure.* After 9 days skin soft and velvety, no blood stains on wrists. After 2 weeks scurfy patches much improved, though persisting. After 3 weeks skin healthy and clean; new fur growing. Penis still dropped

insufficiency of vitamin B_2, but to lack of a further, at present unknown factor, which accompanies vitamin B_2 in many foodstuffs, but is absent from, or deficient in, egg-white [see Chick and Copping, 1930, 3].

The remaining observations in Table I illustrate the swift and certain action of egg-white concentrates in curing the rat dermatitis developed after long periods (13–30 weeks) of vitamin B_2 deprivation. In most cases the skin was restored to a normal state in about 3 weeks with doses equivalent to 5–10 g. of the fresh egg-white. Restoration of growth invariably accompanied healing of the skin lesions, but was usually checked after 2 or 3 weeks.

Attempts at further purification of vitamin B_2 preparations from egg-white.

Alcohol fractionation was tried [see Narayanan and Drummond, 1930; Chick and Copping, 1930, 2], but the precipitate (ovomucoid) formed in alcohol of 70 % strength (by weight) and the filtrate, both proved to be inactive. Using weaker alcohol of 50 % strength, both precipitate and filtrate were found to contain a portion of the original activity. An attempt to make an extract from egg-white by boiling in 50 % alcohol at p_H 4·0 also failed; in this case the filtrate after separation of the precipitated proteins was inactive.

Precipitation with basic lead acetate [Chick and Roscoe, 1929, 2; Narayanan and Drummond, 1930] at p_H 8·0 was also tried, but the solution obtained after decomposition of the precipitate by H_2S was inactive.

Separation of vitamin B_1 from the white in the egg.

The agency by which the vitamin B_1, abundantly present in the yolk, is prevented from diffusing into the surrounding egg-white, is unknown, since both are water-soluble and capable of free diffusion through membranes. A mixture of vitamins B_1 and B_2 contained in a watery yeast extract was found to dialyse freely through a cellophane membrane [Chick and Roscoe, 1929, 2]. It was conceivable, however, that in egg-yolk vitamin B_1 might exist in some combination which prevented free diffusion. Undiluted egg-yolk was therefore placed in a cellophane membrane surrounded by distilled water and left to stand for 4 days at about 0°, but vitamin B_1 was found present in the surrounding fluid.

It is probable that the diffusion of vitamin B_1 in the egg from the yolk to the white is prevented by some property of the living yolk membrane.

Summary.

1. Egg-white of hen's eggs, while a relatively rich source of the anti-dermatitis vitamin B_2, is lacking in vitamin B_1, and in this respect is unique among the foodstuffs hitherto examined.

2. Convenient vitamin B_2 concentrates can be prepared from egg-white by removal of the heat-coagulable proteins. Growth can be restored in young rats suffering from vitamin B_2 deficiency, and the characteristic skin lesions

healed, by daily doses of this concentrate equivalent to 5–10 g. of the original egg-white.

3. Attempts to purify these concentrates further by alcohol fractionation or precipitation with lead acetate have been unsuccessful.

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CXCVII. THE DISTRIBUTION OF THE VITAMIN B COMPLEX.

I. LEAFY VEGETABLES.

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The work described in this paper is a continuation of that already carried out in this laboratory [Aykroyd and Roscoe, 1929] on the distribution among foodstuffs of the two constituents of the vitamin B complex, vitamins B_1 and B_2[1].

The greater part of the early work on the distribution of vitamin B needs reconsideration since the differentiation of the complex into the two constituents, vitamins B_1 and B_2. Some deductions can however be made from this work.

The classic work on the antineuritic vitamin, using pigeons and fowls, remains uncomplicated by any considerations of vitamin B_2 deficiency, since it is concerned purely with the cure and prevention of polyneuritis. Thus both McCollum and Kennedy [1916] and Chick and Hume [1917] showed that preparations from cabbage will cure polyneuritis in pigeons. But no quantitative results can be drawn from their figures.

Osborne and Mendel [1919, 1, 2; 1920] in their investigation of the vitamin B content of green vegetables used as protein an extracted meat residue, which from their control experiments [Osborne and Mendel, 1917] appears to have contained vitamin B_2, though this was not then recognised, but to have been devoid of vitamin B_1. Their results can therefore be interpreted as an estimation of vitamin B_1. They found that 0·5–1 g. of alfalfa, clover or spinach, 1 g. of cabbage or more than 1 g. of timothy grass, all reckoned on the dry weight, was necessary daily to promote the growth of young rats, 0·2 g. being the amount of dry yeast needed.

Dunham [1921] and Sherman [1926] used maintenance, not gain in weight, as their criterion. Since vitamin B_2 is not necessary for weight maintenance, their results may also be interpreted as vitamin B_1 estimations. Sherman found that 0·1–0·15 g. dry weight of spinach, 0·15–0·3 g. of cabbage, 0·2–0·3 g. of lettuce or 0·3 g. of onion was needed daily for the weight maintenance of young rats. The figures given by Dunham were lower, *viz.* 0·07 g. for cabbage and 0·085 g. for spinach, but, as coprophagy was not prevented, the rats probably acquired some vitamin B from their faeces.

[1] For the nomenclature used in this paper see footnote, p. 1764.

Other workers give estimates of vitamin B which can only be interpreted as showing amounts, not necessarily minimal, which provided adequate quantities of both vitamins B_1 and B_2 for the normal growth of the young rat. Thus rats grew normally when the following amounts (dry weight) were fed: 20 % of cabbage in the diet [Daniels and McClurg, 1919], daily doses of 0·4 g. of lettuce and parsley, more than 0·6 g. of dandelion leaves, 1·0 g. of onion and 1·0–2·0 g. of beet leaves [Osborne and Mendel, 1919, 2; 1922], 0·5–2·0 g. of spinach [Eddy, Kohman and Carlsson, 1925], and 0·4 g. of watercress [Mendel and Vickery, 1929].

Salmon [1927] distinguished between vitamin B_1 and vitamin B_2, using pigeon maintenance and rat maintenance in his vitamin B_1 estimations, and rat growth in his vitamin B_2 estimations. The latter observations may therefore be influenced by the vitamin B_1 content, but with the green leaves he tested it is doubtful if this was the case. He found that a daily dose of the equivalent of 0·35–0·4 g. dry weight of rape or velvet bean leaves was sufficient for maintenance of the pigeon, 0·1–0·15 g. dry weight of rape leaves lengthened the life of the rat and 0·25 g. dry weight of velvet bean leaves doubled it. 1–2 g. dry weight of rape leaves or velvet bean leaves was sufficient for good rat growth as against 0·4–0·5 g. of dry yeast.

These results indicate that the vitamin B_1 and vitamin B_2 values of the leafy vegetables are both high, being from one-half to one-fifth that of yeast, when dry weights are considered.

In the present paper vitamin B_1 and vitamin B_2 estimations of watercress, lettuce, cabbage, spinach and onion are reported.

Methods.

The methods employed were those described by Chick and Roscoe for vitamin B_1 [1929] and vitamin B_2 [1928] in which the criterion was the growth of the young rat. The rats received the experimental diet when just weaned and weighing about 40 g. They were fed for a preliminary week on a basal diet (P2L, Table I) deprived of both B vitamins.

Vitamin B_1. The estimations of vitamin B_1 were made in duplicate, using the two vitamin B_2-containing diets previously described by Chick and Roscoe [1929].

In one (EL, Table I) the protein and vitamin B_2 were provided by egg-white, in the other (FL) the protein was supplied as caseinogen and the vitamin B_2 as an autoclaved yeast extract (F5A, Table I). The diet FL differed from that used by Chick and Roscoe (P2L, 1929) in the substitution of "light white casein" (B.D.H.) for the highly purified caseinogen. It was found, following the work of Coward *et al.* [1929], that the former product contained no appreciable amounts of vitamin B_1.

The autoclaved brewer's yeast used previously as source of vitamin B_2 was found to have two disadvantages. It was apt to cause diarrhoea, and some batches, although autoclaved under apparently similar circumstances, appeared to contain small quantities of vitamin B_1, with the result that the time taken by the rats to become depleted of their stores of this vitamin was uncertain and sometimes long. In the present work vitamin B_2 was therefore given as daily doses, equivalent to 0·5 g. of dry yeast, of an extract made from washed brewer's yeast by boiling with 0·01 % acetic acid and autoclaving for 5 hours at 120° (p_H *ca.* 5·0).

When thus provided with vitamin B_2 the rats, presumably because they had not exhausted their stores of vitamin B_1, continued to grow. The rats on the caseinogen and yeast extract diet took slightly longer (about 18 days) to become stationary in weight than those on the egg-white

diet (about 15 days). When their weight had been stationary for a week the rats received doses of vitamin B_1-containing substances.

Table I. *Diets employed in the estimation of vitamins B_1 and B_2.*

Diet	Protein g.	Rice starch g.	Arachis oil g.	Salt mixture* g.	Distilled water cc.
P2L	Purified caseinogen 100	300	75	25	500
	Used (*a*) as basal diet devoid of all B vitamins; (*b*) in vitamin B_2 tests, with the equivalent of 0·6 g. dry yeast of Peters's antineuritic concentrate (YE) daily as source of vitamin B_1.				
FL	"Light white casein" (B.D.H.) 100	300	75	25	500
	Used in vitamin B_1 tests, with a daily dose equivalent to 0·5 g. dry yeast of an autoclaved yeast extract (F5A) daily.				
EL	Egg-white 800 (Protein 100, Water 700)	300	75	25	50
	Used in vitamin B_1 tests, the vitamin B_2 being present in the egg-white.				

All diets were cooked, after mixing, in a steamer for 3–5 hours according to quantity. 0·05–0·1 g. of cod-liver oil was given daily to all rats to provide vitamins A and D.

* No. 185. McCollum, Simmonds and Pitz [1917].

Vitamin B_2. The diet used was P2L (Table I). At the end of one week of total depletion of B vitamins the rats received doses of the vitamin B_2-containing substances and at the same time 0·1 cc. (equivalent to 0·6 g. dry yeast) daily of Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925].

A control rat from each litter received vitamin B_1 only, to ensure that no appreciable growth took place.

In addition to these experiments, in which the criterion for vitamin B_2 was growth promotion, the curative effect on the dermatitis which developed in the vitamin B_2-deficient rats was also observed. Comparable results were however difficult to obtain owing to the variability in the time of onset of symptoms and in their nature and degree of severity.

In the growth tests rats were observed for 5 weeks after receiving their doses of vitamin B_1- or B_2-containing substances and the vitamin content was then estimated by comparing the amounts needed to stimulate an increase in weight of 50–60 g. in 5 weeks with the amount of yeast required.

Materials tested.

The following vegetables were tested for their vitamin B_1 and vitamin B_2 content respectively.

1. Watercress.
2. Lettuce, cabbage variety. These leaves were pale green.
3. Cabbage. Dark green leaves of different varieties were used without distinction. The etiolated inner leaves of the white-hearted varieties were tested separately.
4. Spinach (*Spinacea oleracea*).
5. Onion (Spanish variety). The onion bulb is morphologically comparable with the other vegetables tested.

A few experiments were carried out with fresh samples, but for the most part dried samples were used. These were prepared by plunging the leaves,

previously washed and freed of stalks and midribs, in boiling water for a minute (this by disorganising the cells accelerates the drying), spreading on sheets of paper and drying at 37° for 48 hours. They were then ground to a fine powder in a coffee mill. The onion was scalded, minced and dried for about 96 hours at 37° on metal sheets before grinding.

The fresh watercress, lettuce and cabbage yielded 9 % of dried material, the spinach 11 % and the onion 6 %.

1. *The vitamin B_1 content of the vegetables.*

The results of these experiments are given in Table II. The amounts of the vegetables are expressed as dry weight daily doses.

Table II. *Vitamin B_1 content of vegetables as demonstrated by the weight increase of rats during 5 weeks on a basal diet, complete, except for vitamin B_1, together with a daily dose of the vegetable to be tested.*

Four rats received each dose, two on the egg-white diet (EL) and two on the caseinogen and autoclaved fraction from yeast diet (FL.F5A).

Material tested	Dry weight g.	Increase in weight in 5 weeks (averages g.) Diet EL	Diet FL.F5A	Average
Watercress	0·3	70	93	81
	0·5	95	77	86
Lettuce	0·15	9	8	8
	0·3	49	58	53
	0·5	85	99	92
Cabbage, etiolated leaves	0·3	32	54	43
	0·5	66	72	69
Cabbage, dark green leaves	0·3	38	29	33
	0·9* (10 %)	—	—	85
Spinach	0·5	63	53	58
Onion	0·5	(9)	35	35
	1·0	57	59	58

* The dark green cabbage was in this case the sole source of the vitamin B complex.

Watercress and lettuce. Among the vegetables tested watercress was the richest source of vitamin B_1, an amount small enough to limit growth was not found, 0·3 g. being the least tested. Lettuce came next, rats receiving 0·3 g. gained 53 g., in 5 weeks.

Cabbage. Etiolated cabbage leaves came next, rats receiving 0·3 g. gained 43 g., those receiving 0·5 g. gained 69 g. Dark green cabbage leaves appeared slightly inferior to the white ones.

Spinach. Rats receiving 0·5 g. of spinach gained 58 g. in weight in 5 weeks, this being slightly less than was obtained with the same dose of etiolated cabbage leaves.

Onion. Onion was found to be a poorer source of vitamin B_1 than the foregoing vegetables. The rats receiving 1 g. gained 56 g., the same as those receiving 0·5 g. of spinach.

These results are summarised in Table IV A where the amounts needed to give 50–60 g. increase in weight in 5 weeks are compared.

The above leafy vegetables may be reckoned rich sources of vitamin B_1, being, when dry weights are compared, about one quarter to one-half as rich as yeast, wheat germ or ox-liver [Chick and Copping, 1930], nearly twice as good as egg-yolk [Chick and Roscoe, 1929], and two to three times better than ox-muscle [Chick and Copping, 1930]. When a comparison of their wet weights is made, they take a lower place owing to their high water content.

As far as any comparison can be made between the different standards adopted, the vitamin B_1 values here recorded are of the same order of magnitude as those of Osborne and Mendel [1919, 1, 2; 1920] and of Sherman [1926]. These workers found however that spinach was richer in the antineuritic vitamin than cabbage.

2. *A third factor affecting the growth of rats.*

Chick and Roscoe [1929] showed that in some cases better growth was obtained with rats receiving a caseinogen and autoclaved yeast diet than with those receiving an egg-white diet, although the dose of vitamin B_1 was the same. A further discussion of this problem is given in the paper by Chick and Copping [1930] and an explanation is put forward, that some dietary factor other than vitamin B_2, provisionally called "factor Y," is present in autoclaved yeast but absent from egg-white.

If in the above experiments the growth be compared when the two different diets were used, it will be seen that no difference in the results was apparent when the rats received doses of watercress, dark green cabbage leaves or spinach. A difference so slight as to be problematic was observed with doses of 0·3 g. and 0·5 g. of lettuce and etiolated cabbage leaves and a more marked difference with doses of 0·5 g. of onion, which disappeared when the dose was doubled.

This result is interpreted to indicate that the unknown dietary factor Y is provided in adequate amounts in watercress, dark green cabbage leaves and spinach, present in rather less amounts in lettuce and etiolated cabbage leaves and in much smaller amounts in onion.

3. *The vitamin B_2 content of the vegetables.*

The results of these experiments are given in Table III. The amounts of the vegetables are expressed as the dry weights eaten daily.

Watercress and spinach. These had similar vitamin B_2 contents, rats eating 0·4 g. in each case increased in weight by the same amount, 55 g. in 5 weeks. Watercress may be slightly better than spinach as rats receiving 0·2 g. also gained 53 g.

Lettuce. This appears to be slightly poorer in vitamin B_2 than watercress and spinach; rats receiving 0·6 g. gained 55 g.

Cabbage. The dark green leaves of the cabbage had the same vitamin B_2

content as watercress or spinach, rats eating 0·4 g. gained 58 g. in weight. The etiolated leaves, however, were poorer even than lettuce, with doses of 0·8 g., 65 g. increase in weight was observed, but only 36 g. with doses of 0·4 g.

Table III. *Vitamin B_2 content of vegetables and yeast as demonstrated by the weight increase of rats during 5 weeks on a diet (P2L. YE) complete except for vitamin B_2, together with a daily dose of the vegetable to be tested.*

Material tested	Dry weight g. daily	No. of rats	Growth in 5 weeks Averages g.
Yeast	0·1	4	50
	0·2	1	75
	0·25	2	85
	0·4	2	110
	10 %≡1·0*	4	110
Watercress	0·2	3	53
	0·3	3	57
	0·4	5	56
Lettuce	0·2	2	44
	0·3	5	46
	0·6	4	57
Cabbage, etiolated leaves	0·2	2	34
	0·3	3	35
	0·4	4	36
	0·8	4	65
Cabbage, dark green leaves	0·2	2	41
	0·4	5	58
	10 %≡0·9*	2	85
Spinach	0·2	3	41
	0·4	5	54
	0·6	4	67
	0·8	4	83
Onion	0·8	2	21
	25 %≡1·6*	4	53

* The foodstuffs were in these cases given as a percentage of the diet.

Onion. This was the poorest source of vitamin B_2. Rats gained only 20 g. in 5 weeks when consuming 0·8 g. daily, or 53 g. with 1·6 g. (given as 25 % of the diet). Larger doses were not tried.

Yeast. In Table III are also given figures for dried brewer's yeast. With daily doses of 0·1 g. the average increase in weight was 50 g. in 5 weeks.

These results are summarised in Table IV B where the amounts needed to provide enough vitamin B_2 for 50–60 g. increase in weight in 5 weeks are compared.

The green vegetables tested were from one-eighth to one-half as rich in vitamin B_2 as dried yeast. They are thus poorer than ox-liver, about equal to milk, steak or egg-yolk and considerably better than the cereals or pulses [Aykroyd and Roscoe, 1929], these comparisons being made on the dry weights. If their wet weights are considered, however, they are inferior even to the cereals, though still equal to milk.

In Table V are shown the amounts of these vegetables needed to cure dermatitis developed in rats on a diet deficient in vitamin B_2. It will be seen that daily doses of 0·3 g. of dry watercress, slightly more than 0·2 g. of spinach,

Table IV. *The vitamin B_1 and B_2 contents of certain vegetables as compared with that of yeast.*

Minimal amounts needed daily to promote an increase in weight of 50–60 g. in 5 weeks in young rats of 40 g. weight.

Material	A As source of vitamin B_1		B As source of vitamin B_2	
	Dry weight g.	Wet weight g.	Dry weight g.	Wet weight g.
Yeast	0·1?	0·5?	0·1	0·5
Watercress	< 0·3	< 3·75	0·2–0·4	2·5–5·0
Lettuce	0·3	3·75	0·6	7·5
Cabbage, etiolated leaves	0·3–0·5	3·75–6·25	0·4–0·8	5·0–10·0
Cabbage, dark green leaves	> 0·3–0·5	> 3·75–6·25	0·4	5·0
Spinach	0·5	4·5	0·4	3·6
Onion	1·0	16·6	1·6	26·6

0·4 g. of lettuce and 0·5 g. of etiolated cabbage leaves were needed to cure these symptoms. With onion in one case a daily dose of 0·5 g. appeared to cure, but in a more severe case no cure was obtained with 0·8 g. daily.

Table V. *Vitamin B_2 content of vegetables as demonstrated by the curative effect of the vegetables when fed to rats which had developed dermatitis on a diet devoid of vitamin B_2 (P2L. YE).*

Material tested	Dry weight g.	Rat No.	Days on deficient diet	Symptoms	Cure
Watercress	0·2	15070 ♂	42	Eyes closed, fur on face poor. No skin symptoms	Condition became worse
	0·4		49	Eyes very bad, fur very poor	Cured in 3 weeks
	0·3	7005 ♂	140	Inflamed patch on ear. Eyes slightly spectacled	Cured in 3 weeks
Lettuce	0·2	9021 ♂	82	Eyes nearly shut, spectacled. Fur poor	Further symptoms developed
	0·4		98	Skin peeling round eyes and on face, very bad state	Cured slowly
	0·3	15089 ♀	75	Eyes very bad, fur poor	No improvement
	0·4		82	Nose and face losing hair	Cured in 3 weeks
Cabbage, white	0·4	12043 ♂	98	Eyes nearly shut, fur on face poor. No skin symptoms	Eyes cured
	0·3	18097 ♂	63	Skin of one finger on each hand affected. Fur poor	No improvement
	0·5		75	Symptoms as before and losing hair round mouth	Cured in 3 weeks
Spinach	0·2	14061 ♀	123	Eyes spectacled. No skin symptoms	Eyes cured in 3 weeks
	0·2	26157 ♂	105	Sores on abdomen. Fur poor	No cure in 3 weeks
Onion	0·5	15078 ♂	114	Right eye very bad. Coat poor with bald patches, but no inflammation	Eye cured and coat improved but was still not normal
	0·5	24143 ♂	156	Sore patches on head and back. General conditions very bad	General condition became worse. Symptoms the same
	0·8		165	Symptoms as before	Symptoms stationary. General condition very bad. No cure

These figures are only approximate, but it appears that less of the vegetables is needed to cure dermatitis than is needed for good growth. Those that are richest in growth-promoting power have also the strongest curative action.

In addition to the above, an experiment was carried out in which the rats received 10 % lettuce and 10 % dark cabbage, respectively, in the diet as sole source of the vitamin B complex. The growth of these rats for 15 weeks was compared with that of rats receiving 10 % of dry yeast in the diet, and was found to be as good. This would seem to prove that all the factors of the vitamin B complex necessary for the normal growth of the young rat are present in these quantities of lettuce or dark green cabbage leaves.

In order that it might be certain that no portion of the vitamins B_1 and B_2 originally present had been destroyed in the preparation of the dried samples, control rats were fed with the fresh leaves. No appreciable difference was observed either in the vitamin B_1 or B_2 content. The samples were not kept for more than about 2 months, and in this time no deterioration in the content of either vitamin was observed.

The above results suggest that the vitamin B_2 content of the vegetables tested varied with the degree of greenness, being greatest in the darkest green vegetables and least in the colourless onion. It is possible, however, that the figures obtained for lettuce, etiolated cabbage leaves and onion were to some extent lowered by a deficiency of the third hitherto unrecognised factor Y, which appears to be present in insufficient amounts for good growth in these vegetables and also to be present in only small quantities in Peters's anti-neuritic concentrate (see Section 2).

4. *The effect of cooking on the vitamin B_1 and vitamin B_2 content of spinach.*

The spinach was freed of stalks and midribs, placed in a pan with a little water and boiled for 15 minutes. Much juice comes out in this time, so that the shrunken leaves are finally covered by the liquid; this is drained off by pressing by hand in a colander. The only deviations from the domestic method of cooking were that distilled water was used and no seasoning was added.

Vitamins B_1 and B_2 were estimated in the cooked pressed spinach after drying, and in the juice. The results are summarised in Table VI.

With equivalent doses of the dried cooked spinach and of the juice, given as source of vitamin B_1, the rats gained similar amounts of weight. About half of the vitamin B_1 had presumably passed into the juice. When the cooked spinach and juice were given as source of vitamin B_2, the rats receiving the juice gained more in weight than those receiving an equivalent amount of the cooked spinach. More than half the vitamin B_2 therefore appeared to have passed into the juice. It is however possible that the vitamin B_2 might be more readily absorbed by the rat from the juice than from the solid.

Table VI. *The effect of cooking on the vitamin B_1 and B_2 content of spinach.*

The estimation was made by comparing the growth-promoting power for young rats of daily doses of the dried uncooked spinach and of the dried cooked spinach and the juice from it.

	As source of vitamin B_1			As source of vitamin B_2		
Material	Dose	No. of rats	Average increase in weight in 5 weeks g.	Dose	No. of rats	Average increase in weight in 5 weeks g.
Uncooked	0·5 g. dry*	4	58	0·6 g. dry†	4	67
Cooked	0·5 g. dry†	3	34	0·6 g. dry‡	4	47
Juice	2·0 cc.†	2	30	2·4 cc.‡	2	58

* From 4·5 g. fresh. † From 5·5 g. fresh. ‡ From 6·4 g. fresh.

From these results it appears that in the domestic cooking of spinach half of both B vitamins is lost in the water.

Previous work on the vitamin B content of cooked vegetables has in the main been devoted to the investigation of the destructive action of heat. Thus McCollum and Davis [1915], Chick and Hume [1917] and Sherman and Grose [1923] among other writers showed that cooking for as much as 4 hours at 100° did not materially affect the water-soluble B vitamins.

Dunham [1921], however, investigated the extractive effect of the cooking water when various vegetables were cooked according to domestic methods. His criterion was the maintenance of weight without growth of young rats, and his results may therefore be taken as vitamin B_1 estimations. He found that the following percentages of the vitamin were lost: potatoes, boiled without the skins 48 %, boiled with skins 32 %, baked 32 %; carrots, sliced and boiled 34 %; cabbage, boiled 26·5 %; canned peas, boiled 8 %.

The cabbage tested by him lost only half as much vitamin B_1 as the spinach here tested. This is probably due to the greater loss of juice from the spinach.

Miller [1920] and Whipple [1920] investigated the loss of vitamin in the cooking of carrots, navy beans, cabbage and onion and found that about 50 % passed into the water. Their method was, however, that of yeast-growth, which has since been shown not to be specific for the water-soluble B vitamins necessary for rat growth.

Summary.

1. Methods for estimation of vitamins B_1 and B_2 using the growth of rats as criterion are described. They contain a few modifications of those previously published from this laboratory.

2. Watercress, lettuce, spinach and cabbage (both the outer dark green leaves and inner etiolated ones), when dry weights are considered, have a content of vitamin B_1 and of vitamin B_2 about one-fourth that of dry brewer's yeast. Their content of vitamin B_1 is lower than that of wheat germ or ox-liver and higher than that of egg-yolk or ox-muscle. The content of vitamin B_2 is lower than that of ox-liver, equal to that of milk, ox-muscle or egg-yolk

and higher than that of the pulses or cereals. In their natural states, however, owing to their high water content, they are relatively poor sources of these vitamins.

3. The onion bulb, composed of colourless leaves, is poorer in both these vitamins than the green leaves, having about one-twelfth to one-sixteenth the value of dry yeast.

4. The distribution of vitamin B_2 appears to bear some relation to the greenness of the leaf, being greater in the dark green leaves than in the paler ones. No such difference is found in the case of vitamin B_1.

5. The vegetables tested were on the whole richer sources of vitamin B_1 than of vitamin B_2.

6. In the cooking of spinach by the domestic process about half of both vitamins B_1 and B_2 is lost in the cooking water and juice.

7. Evidence is put forward in confirmation of the theory, that when egg-white is given as source of vitamin B_2, the lack of a third factor (factor Y) necessary for the normal growth of the rat becomes apparent. This factor is present in autoclaved yeast extracts and in all the vegetables here tested, but the amounts present in lettuce and etiolated cabbage leaves are less than in the dark green leaves tested, and the amount present in the onion bulb is still smaller.

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CXCVIII. THE COMPOSITE NATURE OF THE WATER-SOLUBLE VITAMIN B[1].

III. DIETARY FACTORS IN ADDITION TO THE ANTI-NEURITIC VITAMIN B_1 AND THE ANTI-DERMATITIS VITAMIN B_2.

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In 1926 Goldberger and his colleagues [1926] published the discovery of a new constituent, more heat-stable than the antineuritic vitamin (vitamin B_1), in the complex previously known as "water-soluble B" [McCollum and Davis, 1915]. This constituent, now known as vitamin B_2, they called the P-P, or pellagra-preventive, vitamin, adducing much evidence that it was concerned in the prevention and cure of human pellagra.

During the four years that have elapsed since the publication of Goldberger's work, the discovery has been announced of at least three additional water-soluble B vitamins [Williams and Waterman, 1927, 1928; Hunt, 1928; Reader, 1929, 1930; Peters, 1929]. For the separate existence of two of these, the Williams and Waterman vitamin B_3, required for weight maintenance in the pigeon, and the Reader vitamin B_4, necessary for correct nutrition of the rat, there is now an accumulation of confirmatory evidence.

In the course of several years spent on this subject the workers in this laboratory have also become convinced that there is present in yeast at least one water-soluble dietary factor essential for the growth and nutrition of the rat in addition to vitamins B_1 and B_2 and the evidence for this statement is contained in this paper. It is, however, certain that this factor which is provisionally called "Factor Y" is not identical with the new vitamins described

[1] The following nomenclature is here adopted for the B group of water-soluble vitamins.

Vitamin B_1 for the more heat-labile, antineuritic, antiberiberi vitamin, discovered by Eijkman [1897].

Vitamin B_2 for the more heat-stable, "P-P" factor, discovered by Goldberger and others 1926, found necessary for growth and prevention of dermatitis in rats.

Vitamin B_3 for the most heat-labile factor of the group, described by Williams and Waterman [1927], present in yeast and whole wheat and necessary for weight maintenance in the pigeon.

Vitamin B_4, for the heat- and alkali-labile factor described by Reader [1929], present in yeast and necessary for the continued growth of the rat; this factor has been known previously as "vitamin B_3."

respectively by Williams and Waterman and by Reader, seeing that it has been found to be highly resistant to the action of heat and alkali, whereas vitamins B_3 and B_4 are described as relatively sensitive to these agents [Reader, 1929; Eddy, Gurin and Keresztesy, 1930].

Much difficulty and uncertainty in this field of work is due to the fact that, except in work on the antineuritic vitamin B_1 and to some extent in that on the antidermatitis vitamin B_2, the criterion for presence or absence of these various B vitamins has been the growth (increase in weight) of a rat or pigeon. The interpretation of growth observations is more complicated and more liable to error than that of tests involving the cure or prevention of an easily recognised pathological syndrome. Advance in this field of work would be much helped if the physiological rôle of the various members of the group were elucidated and the pathological condition following deprivation recognised.

An unknown dietary factor Y in yeast.

(i) *Evidence derived from long period growth trials on "complete" synthetic diets.*

The existence of an unknown water-soluble dietary factor in yeast was suspected in the course of attempts to rear and breed young rats on artificial diets containing all recognised essential constituents, vitamins B_1 and B_2 being supplied from different sources. When the former was given as Peters's antineuritic concentrate prepared from brewer's yeast [Peters, 1924; Kinnersley and Peters, 1925; Chick and Roscoe, 1929, 2] it was found that the nutritive value of the diet was greater if vitamin B_2 were provided as autoclaved yeast or as an autoclaved watery yeast extract than if it were derived from egg-white, a material which, while rich in vitamin B_2 is devoid of vitamin B_1 [Chick, Copping and Roscoe, 1930].

The two diets were composed as follows. Diet I was the ordinary "– B" basal diet (P2L) of this laboratory. It consisted of purified caseinogen 100; rice starch 300; hardened cotton seed or arachis oil 75; salt mixture [McCollum and others, 1917; No. 185] 25; water 500; and was steamed for 2–3 hours before feeding in order to cook the starch grains and prevent refection. Vitamins A and D were given separately to each rat in daily doses of cod-liver oil, 0·05–0·10 g., according to the weight of the animal. Vitamin B_1 was provided as 0·1 cc. daily (≡ 0·6 g. dry yeast) of Peters's concentrate and vitamin B_2 by 0·4 g. daily of dried brewer's yeast which had been autoclaved for 5 hours at 120° or an equivalent amount of a watery yeast extract, similarly heated. Many of the observations lasted for 6–9 months and these vitamin rations were proportionately increased as the rats grew larger.

The composition of diet II was similar to that of diet I except that the 100 parts of purified caseinogen were replaced by an equivalent amount (800 g.) of ordinary fresh egg-white, which supplied both protein and vitamin B_2. The egg-white was coagulated by heating before mixing with the

other constituents of the diet and as with diet I the mixture was steamed before feeding. Vitamins B_1, A and D were administered to the rats separately in daily doses as to the animals on diet I.

The observations were made on young rats just weaned, 21 days old and of 35–45 g. body weight. During the first week it was usual to give all rats diet I without any addition of B vitamins so that this short period of deprivation might make the rats more eager to take the daily rations of these vitamins when offered subsequently.

Growth on diet I. Out of 11 rats (5 ♂ and 6 ♀) on diet I, the growth of 5 (2 ♂ and 3 ♀) reached the standard of the normal Wistar curve [Greenman and Duhring, 1923] over periods of observation ranging from 18 to 30 weeks. Of the remaining 6 rats, 4 were maintained at a level only slightly below this standard.

The rats were mated when about 4 months old and litters of 6–8 normal young were cast by 3 of the 6 females, and one of these had two successive normal litters. The supply of B vitamins was increased to 3–4 times the usual dose during pregnancy and vitamin E given three times weekly as a small ration of wheat germ oil. Nevertheless, the diet proved inadequate to support lactation; none of the young was reared, all died within 24 hours or were eaten by the mothers.

Growth on diet II. Out of a large number of trials only 5 rats (2 ♂ and 3 ♀) showed sustained growth for periods long enough to be comparable with those on diet I. An example of the usual failure of rats on diet II compared with litter mates on diet I is shown in the curves in Fig. 1. The increase in weight of the two exceptional males which grew well and were observed for over 30 weeks was below the Wistar standard; that of the three females was also subnormal, though not markedly lower than that of some females on diet I. The two better grown of the three females were duly mated but without any successful pregnancy, although the observations lasted till the rats were 8 and 10 months old respectively. In both cases a sudden increase in weight occurred, followed by an unexpected fall, which was suggestive of a miscarriage, but no definite proof was obtained.

It seemed unlikely that the cause for the inferiority of diet II lay in the change of protein from caseinogen to egg-white, for the latter has repeatedly been found satisfactory for the rearing of rats receiving protein from no other source [Osborne and Mendel, 1913; Mitchell, 1925]. Nevertheless, to test this point two male rats were reared for 3 months on the caseinogen diet I with vitamin B_2 supplied as a concentrate prepared from egg-white [Chick, Copping and Roscoe, 1930]. The growth of both was subnormal and inferior to that of the males on diet II.

There was the further possibility that the rats on diet II might suffer from an inadequate ration of vitamin B_2 seeing that this was incorporated in the diet and the intake depended on the appetite. This diet was, however, well taken and the average daily intake was found to contain about 10–15 g. egg-white for rats up to 100 g. weight and about 14–18 g. for those heavier. These

amounts would supply considerable excess of vitamin B_2 [Chick, Copping and Roscoe, 1930].

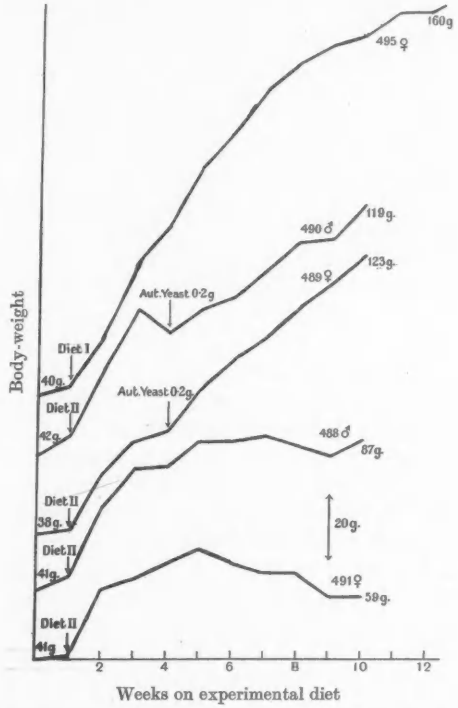

Fig. 1. Showing growth of young rats, litter mates, maintained for one week on a basa diet deprived of B vitamins and thereafter on

(1) Diet I (caseinogen diet) with vitamin B_2 as 0·4 g. daily (later 0·6–1 g.) yeast autoclaved at 120° for 5 hours and vitamin B_1 as Peters's concentrate, 0·1 cc. (≡0·6 g. yeast), later 0·2 cc.; rat 495 ♀.

Or (2) Diet II (egg-white as protein and source of vitamin B_2) with vitamin B_1 as Peters's concentrate: 0·05 cc. daily, rats 490 ♂ and 491 ♀; 0·1 cc. daily, rats 488 ♂ and 489 ♀.

Failure of growth of rats 490 and 489 restored by administration of 0·2 g. autoclaved yeast daily.

The conclusion we are led to adopt from these results and from those given in the following sections is that an essential, water-soluble, heat-stable, dietary factor hitherto unrecognised is present in autoclaved yeast and autoclaved watery yeast extracts, and deficient in egg-white. This dietary factor is provisionally called "Factor Y."

We have no explanation to offer of the fact that diet I, although superior to diet II, is itself unable to maintain normal development indefinitely or to support lactation. We have tried addition of iodine to the salt mixture; a change from purified to unpurified caseinogen ("light white casein", British

Drug Houses) and addition of antiscorbutic vitamin to the diet as a small daily ration of decitrated lemon juice. These changes, however, were without effect in raising the adult body weight of the males beyond about 300 g. or in enabling the females to rear their litters successfully.

Experiences similar to this are common among workers who have used "complete" artificial diets [Palmer and Kennedy, 1927, 1, 2, 1928; Evans and Burr, 1927, 1928; Guha, 1930]. Work is being continued in the hope of discovering the missing essential constituent or constituents and the natural foods in which these are contained.

(ii) *Evidence derived from observations on previously deprived animals.*

(*a*) *Experiments with rats deprived of the antidermatitis vitamin B_2.* The inability of vitamin B_2 contained in egg-white or its products to supplement the antineuritic vitamin B_1 in maintaining steady growth is more strikingly demonstrated when it is given to young rats which have previously been deprived of vitamin B_2 and have received vitamin B_1 in a purified form (Peters's antineuritic concentrate). Whether the period of deprivation is only of a few weeks' duration or long enough for development of the dermatitis caused by vitamin B_2 deficiency, restoration of growth is immediate, and in the latter case rapid cure of dermatitis also takes place. But the rate of increase in body weight soon diminishes and is not restored by increasing the dose of egg-white [Chick, Copping and Roscoe, 1930].

In such experiments the deficiency in egg-white is more clearly demonstrated than in those described in Section (i) above. This is probably due to exhaustion of the young rats' reserves of "factor Y" during the previous period of deprivation, for the Peters's antineuritic concentrate given to provide vitamin B_1 appears to contain little if any of this factor (see below).

(*b*) *Experiments with rats previously deprived of the antineuritic vitamin B_1.* In a previous paper [Chick and Roscoe, 1929, 1] methods were described for estimation of vitamin B_1 in foodstuffs, in which the growth of young rats was taken as a criterion. Young rats were "run out" until the weight became stationary on diets deprived only of this vitamin, and comparative tests were made using the two basal vitamin B_2-containing diets, I and II, described in the previous section. The content of vitamin B_1 in the test material then administered was measured by the degree to which growth was restored during an observation period of 2–5 weeks. It was found that in testing some materials concordant results were obtained with the two different basal diets, with others the degree of growth restored on diet II (egg-white) was consistently inferior.

Further evidence of this discrepancy is contained in the work of Roscoe [1930] on the vitamin B_1 content of vegetables and in some additional data contained in Appendix I. These results may be summarised as follows.

(1) In growth tests with rats devised to estimate the vitamin B_1 contained in dried yeast and certain watery yeast extracts, in ox-liver, egg-yolk, and green

vegetables, watercress, lettuce, spinach, cabbage, it was found immaterial whether vitamin B_2 were supplied in the basal diet as autoclaved yeast products (diet I) or as egg-white (diet II).

(2) With meat (ox-muscle), wheat embryo and the etiolated inner leaves of cabbage, there was consistently inferior growth on diet II.

(3) In tests of onion and Peters's antineuritic "B_1" concentrate, concordant results were obtained if a large ration were given, but with small doses the growth on diet II was always inferior.

These results can be explained by the theory advanced in the previous section, that there is an essential dietary factor, deficient in egg-white but present in yeast and yeast extracts, and also present in adequate amount in the foods of group (1) and present in less amount or lacking in those of groups (2) and (3). The distribution of the unknown factor Y, as thus far studied, indicates its presence in foods known to possess the highest general nutritive qualities.

(iii) *Further study of factor Y as it occurs in yeast extracts.*

Reader [1929] in her work on the B group of vitamins found that antineuritic vitamin B_1 concentrates prepared from yeast by the Peters process were freer from admixture with her "vitamin B_4" if the final solution from the norite adsorbate were made with $N/10$ HCl instead of with 50 % acid alcohol. The vitamin B_1 preparations hitherto used in our work were prepared by the latter method and although the available evidence was opposed to the idea that "vitamin B_4" and factor Y might be identical, seeing that the former is relatively heat-labile, it seemed worth while, nevertheless, to investigate the two types of vitamin B_1 concentrate, to see if any difference could be detected in their ability to supplement vitamin B_2 when presented respectively as egg-white or as autoclaved yeast extract.

Extracts were, therefore, made from aliquot portions of the norite adsorbate derived from yeast XXVI with (1) $N/10$ HCl and (2) 50 % alcohol containing 1 % HCl. In each case the extractions were made by boiling with successive quantities of fresh reagent, these being afterwards combined and concentrated under reduced pressure until 1 cc. contained the equivalent of 6 g. yeast. The reaction was then adjusted to p_H 3·0 for storage.

Prof. Peters warned us that the vitamin B_1 content of extract (1) would probably be much lower than that of (2), and kindly estimated the antineuritic value of both by curative tests on pigeons suffering from polyneuritis. As was predicted, the alcoholic extract (2) was found to be about six times as potent as the hydrochloric acid extract. Accordingly, in the subsequent tests made to compare the power of these two preparations to supplement vitamin B_2 as provided by egg-white in diet II and by autoclaved yeast extract in diet I respectively, the doses given of (1) and (2) were as 5:1, these quantities being reckoned on the dry weight of the equivalent yeast.

The results of a series of such tests are given in Table I. Young rats,

Table I. *Effect of vitamin B_1 contained in extracts made (1) with N/10 HCl and (2) with acid 50 °/o alcohol in Peters's process as supplement for vitamin B_2 contained:—(a) in diet I as extract A (watery extract obtained from washed yeast XXIV autoclaved 5 hours at 120°, p_H 5·6, daily dose ≡ 0·5 g. yeast); (b) in diet II as fresh egg-white incorporated in the diet.*

Rat 698 A in Exp. 2 and rats 740, 742 and 739 in Exp. 3, received later a daily dose (≡ 0·5 g. yeast) of extract B (watery extract from yeast XXIV autoclaved in alkaline solution, 4 hours at 119° at p_H 10·3–9·6).

Material used as source of vitamin B_1	Dose cc.	Equivalent in dry yeast g.	Diet	Litter	Rat	Duration of preparatory period, days	Duration of test, days	Body weight: Initial g.	Body weight: Increase during period of test g.	Body weight: Weekly average increase g.	After administration of extract B: Duration of test, days	After administration of extract B: Body weight: Initial g.	After administration of extract B: Body weight: Increase during period of test g.	After administration of extract B: Body weight: Weekly average increase g.
Exp. 1.														
(1) HCl extract	0·25	1·5	I	1698	693 ♀	14	35	48	68	13·5	—	—	—	—
(2) Alc. „	0·05	0·3	„	„	691 ♂	37	„	69	61	12	—	—	—	—
Exp. 2.														
(1) HCl extract	0·25	1·5	„	1730	700 ♀	18	28	52	47	12	—	—	—	—
(2) Alc. „	0·05	0·3	„	„	702 ♂	21	„	55	34	8·5	—	—	—	—
(1) HCl „	0·25	1·5	II	„	695 A ♀	23	„	51	22	5	—	—	—	—
„ „	0·5	3·0	„	„	697 A ♂	18	„	54	33	8	—	—	—	—
(2) Alc. „	0·1	0·6	„	„	696 A ♂	18	„	58	24	7·5	—	—	—	—
„ „	0·1	„	„	„	698 A ♀	21	„	58	37		28	95	57	14
Exp. 3.														
(1) HCl extract	0·25	1·5	I	1783	736 ♂	16	35	41	58	12·5	—	—	—	—
„ „	„	„	„	„	737 ♂	„	„	47	68		—	—	—	—
(2) Alc. „	0·05	0·3	„	„	735 ♀	19	„	44	67	12	—	—	—	—
„ „	„	„	„	„	738 ♂	16	„	41	53		—	—	—	—
(1) HCl extract	0·5	3·0	II	„	740 ♂	„	„	46	51	11·5	14	97	39	—
„ „	„	„	„	„	742 ♂	„	„	„	64		14	110	31	17·5
(2) Alc. „	0·1	0·6	„	„	739 ♀	14	„	42	37	7·5	14	83	39	19·5

21 days old, just weaned, of about 40 g. weight, were "run out" on diets I and II respectively, as in the preparatory period of the tests for estimation of vitamin B_1. When weight was stationary, usually after 2–3 weeks, vitamin B_1 was supplied as appropriate doses of extracts (1) and (2) respectively.

The results showed that rats maintained on the same diet, whether I or II, responded in a similar manner, as measured by the degree of restoration of growth, to each of the two "B_1" extracts. The difference, if any, was in favour of those receiving the concentrate prepared with hydrochloric acid (Table I, *cf.* Rats 693 and 691, Exp. 1; 700 and 702, Exp. 2; 740, 742 and 739, Exp. 3). It is possible that the dose of extract (1), as calculated from the pigeon trials, was overestimated and that excess of B_1 was administered.

The rats maintained on diet II, however, compared with their litter mates on diet I, showed a consistently lower response to both the vitamin B_1 preparations. In Exps. 2 and 3 it was found necessary to double the doses for rats on diet II, in order to get a comparable response. There was, however, no indication that the extracts prepared with alcohol had any supplementary power for egg-white greater than those made with hydrochloric acid.

Table II. *Capacity of extract B, a watery yeast extract (XXIV) deprived of vitamin B_2 by autoclaving (119° for 4 hours) in alkaline solution at p_H 10·3–9·6 (daily dose ≡ 0·5 g. yeast) to supplement egg-white used as source of vitamin B_2.*

Vitamin B_1 provided as Peters's purified antineuritic concentrate (alcoholic extract from norite).

Diet I, caseinogen as protein and vitamin B_2 as extract A (see Table I).

Diet II, protein and vitamin B_2 as egg-white incorporated in the diet.

In Exps. 4 and 5, first period, the dose of vitamin B_1 was 0·045 cc. (≡0·27 g. yeast) and the period of the test 5 weeks; in Exp. 4, second period, the dose of vitamin B_1 was doubled and the test period reduced to 4 weeks.

Exp.	Diet	Litter	Rat	Preliminary period without vitamin B_1 days	Body weight				
					Initial g.	Increase during period of test g.	Average increase weekly g.	Increase during period of test g.	Average increase weekly g.
					First period			Second period	
4	I	1770	727 ♀	25	56	35		33	
			728 ♂	28	63	40	7·5	33	8
	II	„	730 ♂	14	40	34		27	
			731 ♀	14	43	42		37	
			734 ♂	25	45	16	6	24	7
	II + extract B	„	729 ♀	14	39	41		41	
			732 ♀	25	42	37		43	
			733 ♂	14	47	66	9·5	56	12
5	I	1785	743 ♀	14	45	69			
			744 ♂	14	46	68	13·5		
	II	„	750 ♂	23	58	48			
			751 ♂	14	40	50	10		
	II + extract B	„	748 ♀	23	53	63			
			749 ♂	23	58	62	12·5		

(iv) *Supplementary effect of yeast extracts in which both vitamins B_1 and B_2 have been destroyed by autoclaving in alkaline solution.*

At the end of the observations described above certain rats in Exps. 2 and 3 (698A; 740, 742, 739) received a further addition to their egg-white diet, diet II, in the form of a small daily ration (equivalent to 0·5 g. original yeast) of extract B, a watery yeast extract, which had been autoclaved at alkaline reaction. It was prepared as follows. Washed, pressed brewer's yeast XXIV was boiled with twice its weight of 0·01 % acetic acid solution, and the filtrate concentrated until the equivalent of 1 g. yeast (dry weight) was contained in 1·5 cc. Sodium hydroxide was then added until the p_H was 10·3 (determined with a hydrogen electrode) and the material heated in an autoclave for 4 hours at 119°, after which operation the p_H was 9·6. It was immediately brought to p_H 3·0 for storage. This extract will be referred to as extract B. Proof that the vitamin B_2 originally richly present (see extract A, Tables I and II) had suffered destruction by this treatment was shown by the negative results of growth tests and curative trials of dermatitis made on rats suffering from vitamin B_2 deficiency [Chick and Copping, 1930].

This material, however, was found to contain a factor supplementing diet II, for the rats in Exps. 2 and 3 to which it was given responded immediately and increased in weight at rates varying from 14 to 20 g. weekly, whereas previously the rate of increase had been subnormal, from 7·5 to 11·5 g. weekly. This result was confirmed by the results of two additional experiments (4 and 5, Table II), in which young rats were prepared similarly to those in Exps. 1–3 and maintained on diet II, but of these half received a daily ration of extract B from the time when vitamin B_1 was given.

Exp. 4 was divided into two periods, in the first 5 weeks the dose of vitamin B_1 given was sub-optimal and the growth of all the rats was subnormal. The increase in weight of those on diet II without addition of extract B was as usual inferior (average weekly increase 6 g.) to that of those on diet I, but the addition of extract B caused a growth rate (average weekly increase 9·5 g.) superior even to that of those on diet I (average weekly increase 7·5 g.). During a further period of 4 weeks' observation the dose of vitamin B_1 was doubled and growth in all three groups was enhanced, but showed a similar relationship, *viz.* average weekly increase of 8 g., 7 g. and 12 g. on diets I and on diet II without, and with, extract B respectively.

Exp. 5 included 6 rats, litter mates, divided into 3 groups as in Exp. 4. Extract B was given to two rats on diet II during the whole preparatory period, in order to prevent any possible depletion of the reserves of factor Y. After administration of vitamin B_1 growth was observed for 5 weeks. The result confirmed Exp. 4. The average weekly increase in weight of rats on diet I was 13·5 g., and of those on diet II without, and with, extract B, 10 g. and 12·5 g. respectively.

The increased gain in body weight observed when extract B was added to

diet II was related to an increased appetite and intake of food. Thus, in Exp. 3, rats 740 and 742, during 5 weeks on diet II consumed an average of 14 g. food daily; during the subsequent 2 weeks, when extract B was given, the average daily consumption was 21 g. Similarly in Exp. 5 rats 750 and 751, during 5 weeks' observation on diet II, consumed a daily average of 17 g. food, while for their litter mates 748 and 749 which received extract B in addition the daily average was 22 g.

(v) *Nature of factor Y contained in alkaline-autoclaved yeast extract.*

To find an interpretation of the results in the previous section it might be argued that the inferior growth of the rats on the egg-white diet II was caused by insufficient intake of vitamin B_2, which might occur towards the end of tests when appetite flagged, seeing that this vitamin was incorporated in the diet. Yeast extract prepared as extract B by strong heating in alkaline solution has not, however, in our experience, been found to contain appreciable amounts of vitamin B_2. The negative results of the tests carried out in this laboratory with the actual extract B used in Exps. 2–5 have been already published [Chick and Roscoe, 1930; Chick and Copping, 1930], and curative tests on rat dermatitis with a similar preparation, extract BB (used in Exp. 6 Appendix II) have been kindly undertaken by Dr Reader at Oxford, who has confirmed our experience.

Another possible explanation of the beneficial effect of extract B when egg-white is the sole source of protein and nitrogen might be that it provides some nitrogenous material absent from egg-white but present in caseinogen or in the yeast extract A given in diet I to provide vitamin B_2. This nitrogenous material could hardly be of a protein nature, for the coagulable proteins of yeast with isoelectric point at *ca.* p_H 5·0 are removed in the preparation of these extracts by filtration after boiling in the 0·01 % acetic acid solution. It was possible, however, that some extremely stable, non-protein, nitrogenous substance essential for nutrition, might be present in these watery yeast extracts and perhaps also in caseinogen and lacking in egg-white. It has been found however that diets combining protein as caseinogen, vitamin B_2 as protein-free concentrates prepared from egg-white (extract E), with vitamin B_1 as Peters's purified concentrate are no more satisfactory for growth than those in which the two former are supplied as egg-white (see also Exp. 6 in Appendix II).

A remaining possible explanation and the one here adopted is that watery yeast extracts, in addition to vitamins B_1 and B_2, contain a further essential dietary factor, which is stable to severe heating in acid or alkaline solutions.

Stability to heat and alkali of the various B vitamins.

The evidence relating to the stability to high temperature, acid and alkali of the different B vitamins is in part conflicting. This has led to some confusion in the literature and to difficulty in the interpretation of experiments in which heating operations have been used to effect separations and to gain proof of

the separate existence of the various factors. It is evident, from the available information which is collected in Table III, that there is room for more data based on accurate quantitative experiments.

Table III. *Stability to heat and alkali of B vitamins.*

Vitamin	Source	Temp. °C.	Duration of heating (hrs.)	p_H	Result	Nature of test. Experimental animal	Observer
Antineuritic or antiberiberi B_1	Wheat embryo	*ca.* 100	2	—	No significant loss	Cure of polyneuritis in pigeon	Chick and Hume [1917]
	,,	113	0·66	—	50 % loss	,,	,,
	,,	118–124	2	—	75–90 % loss	,,	,,
	Yeast extract (marmite)	100	1	—	*ca.* 50 % loss	,,	,,
		122	2	—	*ca.* 70 % loss	,,	,,
Antidermatitis B_2	Yeast	120	2·5	—	Stable	—	Goldberger *et al.* [1926]
	,,	120	5	—	50 % loss	Growth of rat	Chick and Roscoe [1927]
	Yeast extract	90–100	2	Acid, p_H 5·0	No significant loss	,,	Chick and Roscoe [1930]
	,,	90–100	2	Alk. p_H 8·3	50 % loss	,,	,,
	,,	123	4	Acid, p_H 5·0 or 3·0	50 % loss	,,	,,
	,,	122–125	4–5	Alk. p_H 8·3–10·0	75–100 % loss	Growth and also cure of dermatitis of rat	Chick and Roscoe [1930]; Chick and Copping [1930]
	Yeast extract (marmite)	120	1	Alk. p_H 9·0	Stable	Growth of rat	Reader [1929]
	Yeast extract	110–120	1–3	Alk. 10–15 % $Ba(OH)_2$	,,	,,	Narayanan and Drummond [1930]
Vitamin B_4 (rat)	Yeast extract	120	1	Alk. p_H 9·0	Destroyed	,,	Reader [1929]
Vitamin B_3 (pigeon)	Yeast	20	—	Alkaline	Slowly destroyed	Weight maintenance in pigeon	Eddy, Gurin and Keresztesy [1930]
	,,	60	—	Natural reaction ? p_H *ca.* 5·0	Destroyed	,,	,,
	Malt extract	60		—	,,	,,	,,
Factor Y	Yeast extract	120–125	4	Alk. p_H 9–10	Stable	Growth of rat	Chick and Copping [present work]

The antineuritic vitamin B_1 is generally acknowledged to be relatively labile to heat and alkali, and the antidermatitis vitamin B_2, to be relatively heat-stable at temperatures about 120° in neutral or acid solution. There is disagreement, however, among different observers as to the degree to which this vitamin is sensitive to alkali at this temperature. Vitamin B_3 (Williams and Waterman) is described as being the member of the group most sensitive to heat, being affected at 60°; vitamin B_4 (Reader) has been found to be heat-labile to an extent comparable with vitamin B_1.

The new water-soluble dietary factor Y dealt with in this communication, appears to be the most resistant of the group, for it can survive heating for 4 hours at 120–125° at a p_H of about 10.

Summary.

1. Experiments with young rats are described which show the failure to sustain growth of a diet (diet II) which contains all hitherto recognised necessary constituents, and includes Peters's antineuritic concentrate as source of vitamin B_1 and hen's egg-white as source of protein and vitamin B_2.

2. A similar result was obtained with diets containing purified caseinogen as protein, vitamin B_1 as Peters's concentrate and egg-white, or concentrates prepared therefrom, as source of vitamin B_2.

3. Satisfactory development over long periods, including fertility and successful pregnancy, was attained on a similarly constituted diet, diet I, containing purified caseinogen as protein, Peters's antineuritic concentrate as vitamin B_1, and autoclaved yeast or autoclaved watery yeast extracts (120°; 5 hours; p_H *ca.* 5·0) as vitamin B_2.

4. Diet II was rendered satisfactory for growth by addition of a small daily ration of a watery yeast extract which had been autoclaved in alkaline solution (4 hours; 120–125°; p_H *ca.* 10). In this preparation vitamin B_2 was proved to have been destroyed.

5. It is concluded that yeast and watery yeast extracts contain, in addition to vitamins B_1 and B_2, a hitherto unrecognised dietary factor, referred to in this paper as factor Y, which can withstand prolonged heating in alkaline solution.

6. Comparative experiments with diets I and II carried out by Roscoe [1930] and others, published in an Appendix to this paper, indicate that factor Y accompanies vitamins B_1 and B_2 in nature, is present in relatively large amount in yeast, green leaf vegetables, egg-yolk and ox-liver and absent from, or present in relatively small amount in, egg-white, wheat embryo, meat (ox-muscle), etiolated leaves of green vegetables and onion. Small amounts appear to be present in Peters's antineuritic concentrate as prepared by the authors from brewer's yeast.

7. Factor Y differs from the new members of the vitamin B complex recently described by Williams and Waterman (vitamin B_3) and by Reader (vitamin B_4) in its stability to heat and alkali.

Appendix I.

Table IV. *Influence on growth of young rats, maintained on basal diets I and II respectively, of the addition of vitamin B_1 as contained in different foodstuffs.*

Diet I contained caseinogen as protein and autoclaved (120°, 5 hrs., p_H *ca.* 5·0) yeast or yeast extract as source of vitamin B_2.
Diet II contained egg-white as protein and source of vitamin B_2.

				Basal diet I						Basal diet II					
		Daily dose					Body weight					Body weight			
Exp.	Foodstuff tested	Natural food g.	Dry weight g.	Rat No.	Duration of preparation period. Days	Duration of test. Days	Initial g.	Increase during period of test g.	Average increase g.	Rat No.	Duration of preparatory period. Days	Duration of test. Days	Initial g.	Increase during period of test g.	Average increase g.	Result
1	Dried egg-yolk A	0·95	0·5	461 ♀	21	21	51	9	—	464 ♀	21	21	40	5	—	Growth slightly better on diet I in Exps. 1 and 2; no difference in Exps. 3 and 4
		1·9	1·0	466 ♂	30	28	72	66	—	465 ♀	18	28	38	55	—	
2	Do. B	2·0	1·0	629 ♂	25	35	56	77	—	626 ♂	16	35	56	64		
		,,	,,	643 ♂	26	,,	65	69	—	641 ♂	23	35	54	54		
		,,	,,	651 ♀	25	,,	53	69	72	649 ♂	23	35	50	49	56	
3	Do. F*	,,	,,	*643 ♂	—	14	134	31		*641 ♂	—	14	108	35		
		,,	,,	*651 ♀	—	,,	122	21	26	*649 ♂	—	14	99	16	25	
4	Do. B	3·0	1·5	610 ♀	39	28	80	79	—	620 ♀	16	28	51	79	—	
5	Dried ox-liver III	1·5	0·5	609 ♀	49	35	78	70		628 ♂	15	35	52	87		No significant difference
				631 ♀	20	,,	54	88	79						87	
6	Do.	2·25	0·75	615 ♂	14	28	52	104		606 ♀	17	28	51	79		
									104	617 ♀	14	28	52	88	84	
7	Dried ox-muscle I and II	3·65	1·0	659 ♀	19	35	49	45	—	662 ♂	18	14	56	−11	—	
8	Do.	5·5	1·5	657 ♂	21	,,	60	100		662 ♂	—	35	45	80		Growth on diet II much inferior to that on diet I
		—	—	666 ♂	19	,,	55	87	93	660 ♀	19	,,	48	63		
										669 ♂	17	,,	56	33		
										671 ♀	17	,,	50	43	55	
9	Wheat embryo	—	0·1	607 ♀	49	,,	89	45		608 ♀	17	,,	53	19		
				621 ♀	42	,,	76	40	42·5	618 ♀	14	,,	47	32	25·5	,, ,,
10	Do.	—	0·2	633 ♂	33	,,	62	86		—	—	—	—	—	—	
				634 ♂	33	,,	67	92		632 ♂	19	,,	56	53		
				644 ♀	40	,,	78	60		642 ♀	25	,,	61	41		
				650 ♂	25	,,	47	85		647 ♂	21	,,	49	56		
				652 ♂	37	,,	75	75	80	—	—	—	—	—	50	
11	Peters's antineuritic concentrate Yeast XVIII	0·05 cc. = 0·3 g. dry yeast		550 ♀	47	,,	69	35	—	561 ♀	25	,,	70	28		
				551 ♀	39	,,	59	47		554 ♀	27	,,	64	27	27·5	,, ,,
				562 ♀	42	,,	87	39		553 ♀	25	14	44	15		
				563 ♀	37	,,	77	40	40	559 ♀	25	,,	73	14	14·5	
12	Do.	0·1 cc. = 0·6 g.		—	—	—	—	—	—	†553 ♀	—	35	59	32		
				—	—	—	—	—	—	†559 ♀	—	,,	87	46	39	
13	Do. Yeast XX	0·75	0·45	603 ♀	28	35	67	71		602 ♂	17	,,	51	46		
	Do. Yeast XXIII			630 ♂	14	,,	49	80	75·5	627 ♂	14	,,	53	57	51·5	,, ,,
14	Do. Yeast XX	0·05	0·3	581 ♂	16	,,	55	44	44	585 ♂	20	—	67	24		
										587 ♀	17	—	61	23	23·5	,, ,,

* Tests with freshly prepared sample following the previous ones on same rats.
† Tests following previous ones on smaller dose.

Appendix II.

[Added December 21st, 1930.]

Exp. 6. (See Table V.)

The following additional experiment (Exp. 6) was devised to gain confirmatory evidence upon the various points concerning the existence of Factor Y which are discussed in Section v above. In this experiment comparison was made of the growth of young rats (litter mates) upon the following 6 diets.

1. Diet I (caseinogen) + vitamin B_2 as extract A, watery yeast extract XXVII, autoclaved for 5 hours at 123° at p_H *ca.* 5·0 (daily dose ≡ 0·5 g. yeast).

2. Diet I (caseinogen) + vitamin B_2 as extract E, a protein-free concentrate from egg-white (daily dose ≡ 10 g. egg-white).

3. As (2), but in addition Factor Y given as extract BB, watery yeast extract XXXIII autoclaved 4 hours at 125° at p_H 10·0–9·5 (daily dose ≡ 0·5 g. yeast).

4. Diet II (egg-white).

5. ,, + extra vitamin B_2 as extract E, as in Group 2.

6. ,, + extract BB as in Group 3.

These diets were distributed among 24 newly-weaned rats of body weight *ca.* 40 g., from 3 litters, as shown in Table V. The rats underwent a preparatory period on these diets without any addition, *i.e.* a period of deprivation of vitamin B_1, which varied from 12–17, 17–25, and 17–20 days, respectively, in the 3 litters. Vitamin B_1 was then given as Peters's purified concentrate and caused immediate resumption of growth which was observed for 5 weeks.

The dose of vitamin B_1 was small, 2 drops daily (0·045 cc. ≡ 0·27 g. yeast), to avoid giving any excess which might include an appreciable amount of Factor Y from this source (see p. 1769 above). Nevertheless, in Groups 1, 3, 5 and 6, satisfactory growth occurred during the 5 weeks of observation, the average increase in weight being from 63 to 73 g. or from 12·5 to 14·5 g. a week. In Groups 2 and 4, on the other hand, the increase in weight was markedly less.

In Group 4 the growth was so poor, the average weekly increase being only 7 g., that the observations were only continued for 4 weeks, after which the dose of vitamin B_1 was doubled. The average weekly increase then reached 11·5 g., but it was evident that growth was limited by the supply of Factor Y, for on receiving a daily dose of extract BB these rats showed a dramatic improvement and an average increase in weight of 40 g. in 2 weeks.

A comparison of the rats in Group 4 with those in Group 2 confirms the conclusion stated on p. 1773 above, that the inferior development on egg-white diets is not to be attributed to the nature of the protein given. The rats in Group 2 receiving caseinogen in the basal diet also showed a depressed

* Tests with freshly prepared sample following the previous ones on same rats.
† Tests following previous ones on smaller dose.

Table V. Exp. 6. *Comparison of growth of young rats on "complete" synthetic diets I and II (see p. 1765 above), with vitamin B_1 (? + B_4 Reader) provided as Peters's antineuritic concentrate from yeast and vitamin B_2 from various sources.*

All rats suffered a short preliminary period of vitamin B_1 deprivation after which they received daily:
(*a*) During Period I of 5 weeks, 2 drops daily (0·045 cc. ≡ 0·27 g. yeast) of Peters's concentrate.
(*b*) During Period II of 2 weeks, 4 drops daily (0·09 cc. ≡ 0·55 g. yeast) of Peters's concentrate.

Group	Diet	Litter 1849 Body weight, g. Rat No.	At end of fore period	Increase during Period I	Increase during Period II	Final	Litter 1852 Body weight, g. Rat No.	At end of fore period	Increase during Period I	Increase during Period II	Final	Litter 1865 Body weight, g. Rat No.	At end of fore period	Increase during Period I	Increase during Period II	Final	All litters Av. weight increase, g. Period I Total	Period I Weekly	Period II Total	Av. final body weight
	Diet I (caseinogen)																			
1	+vitamin B_2 as Extract A	763 ♂	52	61	31	144	773 ♂	62	78	34	174	778 ♂	55	99	27	181	73	14·5	25	♂ 166
		764 ♀	49	64	18	131	774 ♀	59	70	18	147	779 ♀	44	66	20	130				♀ 136
2	+vitamin B_2 as Extract E	761 ♂	48	31	19	98	769 ♂	66	54	38	158	—	—	—	—	—	45	9	24	♂ 128
		762 ♀	49	49	22	119	770 ♀	64	47	18	129	—	—	—	—	—				♀ 124
3	+vitamin B_2 as Extract E +Extract BB	—	—	—	—	—	*771 ♂	75	74	46	195	—	—	—	—	—	69	14	31	♂ 195
		—	—	—	—	—	772 ♀	67	64	17	146	—	—	—	—	—				♀ 146
	Diet II (egg-white)																			
4	No addition (vitamin B_2 in diet)	—	—	—	—	—	775 ♂	54	13†	20	87	780 ♂	55	45†	23	123	30†	7	23	♂ 105‡
		—	—	—	—	—	776 ♀	54	39†	23	116	781 ♀	52	22†	25	99				♀ 107‡
5	+extra vitamin B_2 as Extract E	—	—	—	—	—	777 ♂	61	85	30	176	782 ♂	52	70	47	169	70	14	27	♂ 172
		768 ♀	53	55	15	123	—	—	—	—	—	783 ♀	56	72	17	145				♀ 134
6	+Extract BB	765 ♂	60	66	17	143	—	—	—	—	—	784 ♂	52	67	43	162	63	12·5	25	♂ 152
		766 ♀	61	57	20	138	—	—	—	—	—	785 ♀	61	61	20	142				♀ 140

* Had loose stools throughout; was a "grower."
† Experimental period I of 4 weeks only owing to cessation of growth.
‡ These rats showed an average increase of 40 g. during a subsequent extra period of 2 weeks, during which extract BB was given to provide Factor Y.

rate of growth (average weekly increase 9 g.) when vitamin B_2 was provided from an egg-white concentrate. That this was due to lack of Factor Y is proved by the much improved growth when extract BB was given in addition, as in group 3. Here the average weekly increase was 14 g.

The results of Exp. 6 indicate further that egg-white is not devoid of Factor Y but contains it in relatively small amount. The rats in Group 5 which derived all their B vitamins, other than vitamin B_1, from egg-white showed optimal growth during the period of observation, the average weekly increase being 14 g. It is calculated, however, that they ingested daily about 10–15 g. egg-white in their basal diet and the equivalent of 10 g. from the supplementary extract E.

Thus 10 g. egg-white daily appears to be inadequate (Group 2) and 20–25 g. to be necessary (Group 5) to provide sufficient Factor Y to sustain normal growth, whereas an adequate daily dose of Factor Y is contained in 0·5 g. yeast.

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CXCIX. THE BIOLOGICAL VALUES OF PROTEINS.
I. A METHOD FOR MEASURING THE NITROGENOUS EXCHANGE OF RATS FOR THE PURPOSE OF DETERMINING THE BIOLOGICAL VALUE OF PROTEINS.

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The metabolism cages used were those described by Ackroyd and Hopkins [1916] with the detachable wire floor introduced by Boas [1924] and glass separators of Gross and Connell [1923] to effect quantitative separation of urine and faeces.

The method consisted in a determination of the average daily nitrogenous balance sheet on a diet containing a definite proportion of the protein under investigation. Two adult male rats (250 g. weight upwards) were placed in each cage, and determinations made of the total nitrogen ingested and of that excreted in urine and faeces respectively, during a period lasting 4 or 5 days.

The cages of other experimental animals and reagent bottles containing ammonia were removed from the room in which the metabolism cages were kept to avoid any error due to absorption of small quantities of ammonia.

The control of the intake of nitrogen.

The dry constituents of the experimental diet were carefully mixed together and made into a stiff paste with distilled water, samples being removed for determinations of nitrogen. An amount of the diet, more than sufficient for the whole experiment, was put in a wide-necked stoppered bottle, kept in the refrigerator room when not in use. From this stock the approximate amount needed every day was weighed into the daily ration pots, mixed to a creamy consistency with water and placed in the chambers at either end of the two arms of the metabolism cage. The administration of the diet in the form of a liquid paste was a device introduced by Korenchevsky (unpublished experiments) in this laboratory to prevent the scattering of food. Care was taken to gauge the appetite of the rat with sufficient accuracy to avoid a large residue, any traces of food left being collected at the end of the experiment and the nitrogen in it estimated. The diet bottle was weighed before and after the experiment to obtain the total amount of diet removed. The corresponding amount of nitrogen was calculated and this, after correction for the nitrogen contained in the uneaten residue, gave the total intake.

The control of the nitrogen output.

The urine and faeces were collected daily. To prevent decomposition of urine and consequent loss of ammonia 5 cc. of a 5 % solution of carbolic acid and 1 cc. of a 10 % solution of thymol were placed in the beaker used to collect the urine. During the daily collection of excreta and washing of the metabolism cage the rats were placed in small cages of perforated zinc supported by means of zinc arms over large porcelain dishes, from which any urine and faeces passed during this period could easily be collected.

The faeces were transferred daily from the collecting beaker into a weighed porcelain crucible provided with a lid. If soiled with urine, they were previously washed and the washings added to the urine bottle. The faeces crucible, like the urine bottle, was kept in the refrigerator room when not in use. At the end of the experiment the faeces were moistened with a few drops of a 10 % solution of oxalic acid, dried at 110° for 12 hours, cooled in a desiccator, weighed and then powdered with a pestle, the hair often contained being chopped up with scissors to obtain a uniform mixture. The powdered faeces which are extremely hygroscopic were then transferred to a weighing bottle and dried again at 110°. After cooling in a desiccator aliquot portions were weighed out for nitrogen determination.

The metabolism cage, funnel, separator and beakers placed for collection of faeces and urine respectively were washed down daily with dilute acetic acid and distilled water, using a large brush and a glass rod tipped with rubber. The use of acetic acid prevented risk of ammonia loss during the process of washing and helped to remove traces of solid deposit; a little alcohol was also found useful in removing traces of fat. Urine and washings were filtered through muslin into a large weighed stoppered bottle. The hair removed by this filtration was analysed separately for nitrogen, until a number of analyses had shown that the amount concerned was too small to be significant. At the close of the experiment the urine bottle was weighed and aliquot portions removed for nitrogen determinations.

The nitrogen estimations were made by Kjeldahl's method.

Risk of nitrogen loss during the experiments.

It seemed possible that a loss of ammonia from urine might occur owing to the relatively small volume passed (*ca.* 20–30 cc. daily from 2 rats of 300–400 g. weight each) and the large surface of the cage and funnel, both being about 10 in. in diameter. The result of the following test, devised to simulate an actual experiment, showed, however, that no such risk existed. Urine, of known nitrogen content, was allowed to drop from a burette at intervals during the day and trickle over an empty cage, funnel and separator, being finally collected below in a beaker. After 24 hours the whole apparatus was washed as described above and nitrogen estimated in the collected urine and washings. The amount of urine dropped was 50·2 cc., calculated to contain

0·256 g. nitrogen; the nitrogen recovered from the collected urine and washings was 0·256 g.

In a second test 81·2 cc. urine, containing 0·442 g. nitrogen, was allowed to drop at intervals over 3 days, collection of urine and washings taking place daily. The total nitrogen collected was 0·440 g.

Estimation of the biological value of proteins.

The above method can be used to determine the daily nitrogen balance sheet of any pair of rats maintained on a diet containing a known proportion of any given protein (P).

To estimate the biological value of this protein, it is necessary to determine the minimum amount of nitrogen (M) (derived from the given protein as sole source of nitrogen in the diet) which must be absorbed to compensate the daily nitrogenous expenditure (E) on a nitrogen-free diet. The latter is determined for the same pair of rats, being the total nitrogen excreted in faeces and urine on a protein-free diet consisting of fat and carbohydrate only. The biological value of protein P can then be defined as $100 \times \frac{E}{M}$.

The principal technical difficulty lies in the determination of E. Appetite soon fails on diets devoid of nitrogen, sometimes in the first few days of the experiment. It is, therefore, often difficult to secure a sufficient calorie intake to prevent loss of weight. If there should be any significant loss of weight, E will be over-estimated.

The provision of B vitamins is also necessary to maintain appetite and their administration involves the addition of small amounts of extra nitrogen to the diet. By the use of purified concentrates from yeast, however, these additions can be made very small.

A discussion of these complications, together with examples of the calculations involved, are given by Fixsen [1930], who used this method for determining the biological value of purified caseinogen.

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CC. SELECTIVE FERMENTATION.
II. FERMENTATION OF SUGAR MIXTURES BY SAUTERNE YEAST.

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Two strains of "Sauterne" yeast, reported in a preceding communication [Sobotka and Reiner, 1930] preferred glucose to fructose in the fermentation of a glucose-fructose mixture. The value of $K_{G/F}$ [Willstätter and Sobotka, 1922], the measure of this preference, was similar to the $K_{G/F}$ of all other strains of *Saccharomyces* investigated. This was at variance with the findings of several French authors [for references, *cf.* Sobotka and Reiner, 1930], who ascribed to Sauterne yeast a preference for fructose.

We were able to secure a pure culture of authentic Sauterne yeast. This yeast, tested after two passages on a fructose medium, showed a marked preference for fructose ($K_{F/G} = 2$–3), which after five additional passages was greatly increased. The high figures obtained for $K_{F/G}$ ranged from 6 to 12; they show greater variations than the lower values in other experiments for analytical reasons. Besides, $K_{F/G}$ drifted towards lower values during the rapid disappearance of fructose from the fermenting solution.

When this yeast, after twelve passages on fructose, was subjected to two passages on maltose, its $K_{F/G}$ value dropped to 1·15, rising to 3·6 during 5 hours' contact with invert sugar. Thus, the Sauterne yeast may be said to prefer fructose to glucose at an average rate of 4–6, but this ratio is not stable and might readily be changed by adaptation to values varying from little more than unity to very high ones.

When using a mixture of equal amounts of glucose and mannose, $K_{G/M}$ started at 1·5 and dropped to unity. In a fructose-mannose experiment, $K_{F/M}$ varied between 4·25 and 3·15. The figures in both experiments suggest an adaptation to mannose. From $K_{F/G} = K_{F/M}/K_{G/M}$, a $K_{F/G}$ of about 3 might be derived in agreement with the average values obtained in the direct experiment.

Separate fermentations.

The rates of fermentation of glucose, fructose and invert sugar did not differ in most instances more than 10 % from each other with the yeasts previously investigated [Sobotka and Reiner, 1930, Table II]. The rates for the present strain of Sauterne yeast are given in Table II. The differences in

favour of fructose are very pronounced, fructose being fermented more than 20 % faster than invert sugar and from 50 to 100 % faster than glucose.

It is possible that the capacity of this yeast to initiate the alcoholic fermentation of glucose is so much reduced that the cells in a pure glucose solution cannot utilise to their full extent the enzymes involved in the liberation of carbon dioxide and ethyl alcohol. In other yeasts, both glucose and fructose are converted at such a rate into a three-carbon intermediate compound that the latter is supplied in excess to the enzymic apparatus governing the last stages of alcoholic glycolysis.

The bearing of the anomalies in alcoholic fermentation by Sauterne yeast upon other peculiarities in the metabolism of this micro-organism is being investigated.

Experimental.

The methods used were essentially the same as those described in the previous paper [1930]. The medium contained 5–7 % of the sugar, besides 1·0 % KH_2PO_4, 1·0 % peptone, 0·1 % $MgSO_4.7\,H_2O$ and 0·04 % $CaSO_4$. The yeast was transferred every 3 days to fresh media. The method of Bertrand was used for the titrimetric determinations.

Table I gives representative experiments for the fermentation of invert sugar by various specimens of Sauterne yeast. For convenience, the $K_{F/G}$ values were used instead of their reciprocals $K_{G/F}$. The column headed $K'_{F/G}$ gives this value in those experiments in which the value of $K_{F/G}$ changed, signifying an acute change of specificity. While

$$K_{F/G} = \frac{\log f_0 - \log f_n}{\log g_0 - \log g_n}, \quad K'_{F/G} = \frac{\log f_n - \log f_{(n+1)}}{\log g_n - \log g_{(n+1)}}.$$

Exps. 1 and 2 illustrate the increasing preference for fructose.

As the concentration of alcohol is 3 %, after fermentation of the 6 % sugar solution used in these experiments, we tried to ascertain whether or not the drop of $K_{F/G}$ from 12 to about 6 in Exp. 2 was due to the increasing amount of alcohol. Exp. 3 parallels Exp. 2 except that 3 % ethyl alcohol was added at the beginning. Since the selectivity for fructose and its drop during fermentation coincided within the limits of error, we conclude that alcohol has no influence.

Exp. 4 demonstrates the loss of fructose specificity when the yeast was kept in the ice-chest for 3 days after removal of the medium by centrifuging and washing.

Exp. 5 shows adaptation to glucose in a maltose medium, but subsequent re-adaptation to fructose during the invert sugar experiment.

The fermentation of glucose-mannose and fructose-mannose mixtures by crop "Fructose IV" is demonstrated in Exps. 7 and 8. When compared to Lager yeast, glucose and fructose seemed to have changed their rôle. With Lager yeast, $K_{G/M}$ varied between 4·5 and 3·0, while $K_{F/M}$ was little more than unity; with Sauterne yeast, $K_{F/M}$ ranges between 4·25 and 3·15, while glucose is fermented only slightly faster than mannose.

Table I. *Fermentation of sugar mixtures with Sauterne yeast.*

Yeast and temp.	Time min.	Sugar (actual %)	Optical rotation l=2 dm.	Specific rotation	% of total original sugar unfermented: Total sugar	Glucose	Fructose	$K_{F/G}$	$K'_{F/G}$	CO_2 %
1. Sauterne, fructose II and III, 30°	0	6·00	−1·90	−15·8	100·0	51·1	48·9	—	—	0
	90	5·50	−1·50	−13·6	91·7	48·3	43·4	2·10	—	10·7
	215	3·79	−0·23	− 3·0	63·2	38·1	25·1	2·20	—	25·3
	510	1·73	+1·23	+35·6	28·8	25·3	3·5	(3·70)	—	50·9
2. Sauterne, fructose VII, 29°	0	6·29	−2·04	−16·2	100·0	51·2	48·8	—	—	0
	140	4·70	+0·29	+ 3·1	74·8	48·5	26·3	11·45	—	8·0
	290	4·23	+0·96	+11·4	67·3	47·6	19·7	12·60	15·2	18·5
	470	3·12	+1·51	+24·2	49·6	39·7	9·9	6·20	3·7	26·5
	1440	1·02	+1·06	+52·0	16·2	16·2	0·05	(6·00)	—	81·5
3. Sauterne, fructose VII +3% alcohol, 29°	0	6·40	−2·00	−15·6	100·0	51·6	48·4	—	—	0
	140	5·22	−0·11	− 1·6	81·5	50·2	31·3	15·80	—	9·3
	290	4·49	+0·61	+ 6·8	70·2	47·4	22·8	8·90	5·6	20·4
	470	3·35	+1·69	+25·2	52·4	42·2	10·2	7·75	6·9	29·6
	1440	1·72	+1·67	+48·7	26·9	26·2	0·7	(6·25)	—	78·4
4. Sauterne, fructose VII, 3 days in ice-chest, 29°	0	6·00	−2·21	−18·4	100·0	50·3	49·7	—	—	0
	165	4·18	−1·04	−12·4	69·7	38·0	31·7	1·60	—	21·5
	255	3·53	−0·30	− 5·2	58·9	35·0	23·9	2·00	—	35·3
	485	1·48	+0·40	+13·6	22·7	16·5	6·2	1·85	—	57·0
5. Sauterne, maltose II, 27°	0	5·90	−2·26	−19·2	100·0	49·7	50·3	—	—	0
	105	4·64	−1·66	−17·9	78·7	39·8	38·9	1·15	—	14·1
	205	3·91	−0·91	−11·6	66·3	36·4	29·9	1·70	2·9	29·5
	265	3·14	−0·31	− 4·9	53·1	31·6	21·5	1·90	2·4	36·2
	340	2·65	+0·13	+ 2·4	44·9	29·1	15·8	2·20	3·6	44·2
								$K_{G/F}$		
6. Berlin strain, fructose, 27°	0	5·94	−2·44	−20·5	100·0	48·5	51·5	—	—	0
	90	4·71	−2·57	−27·3	79·3	34·7	44·6	2·30	—	15·4
	160	3·76	−2·43	−32·3	63·3	25·4	37·9	2·10	—	28·4
	205	3·08	−2·55	−41·4	51·8	17·5	34·3	2·50	—	37·6
	245	2·60	−2·30	−44·3	43·8	13·8	30·0	2·30	—	45·7
						Glucose	Mannose	$K_{G/M}$		
7. Sauterne, fructose IV, 27°	0	6·55	+4·18	+31·9	100·0	45·7	54·3	—	—	0
	155	5·59	+3·50	+31·3	85·3	37·6	47·7	1·50	—	11·1
	570	3·35	+2·15	+32·1	51·1	23·6	27·5	1·05	—	38·9
	1185	1·63	+1·05	+32·2	24·9	11·5	13·4	1·00	—	59·2
						Fructose	Mannose	$K_{F/M}$		
8. Sauterne, fructose IV, 27°	0	6·23	−4·48	−36·0	100·0	48·7	51·3	—	—	0
	155	5·29	−3·24	−30·6	84·9	36·9	48·0	4·20	—	19·8
	400	3·72	−1·34	−18·0	59·7	18·7	41·0	4·25	—	48·8
	570	2·67	−0·51	− 9·6	42·7	9·9	32·8	3·55	—	61·2
	1185	1·19	+0·14	+ 5·9	19·1	1·6	17·5	3·15	—	72·2

Table II. *Ratio of separate fermentation of hexoses with various specimens of Sauterne yeast.*

Yeast	No.	$K_{F/G}$ (for mixture)	Ratio for separate fermentation: Fructose / Glucose	Invert / Glucose	Fructose / Mannose	Glucose / Mannose
"Fructose II, III"	1	2·15	1·36	1·12	—	—
"Fructose IV"	7, 8	3*	1·75	—	1·30	0·74
"Fructose XVI"	—	>10	2·18	1·61	1·62	0·75
"Fructose XVII"	—	>10	2·18	—	1·27	0·58
"Fructose VII" 3 days in ice-chest	4	1·8	1·46	1·30	—	—
"Maltose II"	5	1·15–2·2	1·53	1·37	—	—
Berlin strain	6	0·44	0·98	1·00	—	—

* Extrapolated from $K_{F/M}/K_{G/M}$.

After the conclusion of this series, several passages were made from the pure culture of Berlin Sauterne used in our previous communication. It had retained its preference for glucose as shown in Exp. 6 of the present Table I.

The rates of separate fermentation from which the figures in Table II are derived were determined in the Kluyver apparatus. The concentrations of sugar (6 %) and yeast (2 % dry weight) were the same as in the experiments of Table I.

SUMMARY.

Genuine Sauterne yeast ferments fructose preferably to glucose in a mixture. $K_{F/G}$ varies from 1·2 to values above 10 depending on adaptation phenomena. It ferments fructose alone 50 to 100 % faster than glucose alone. The mechanism of this anomalous behaviour is discussed.

We wish to express our gratitude to M. l'Abbé Dubaquié for the pure culture of Sauterne yeast.

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CCI. THE NON-SUGAR REDUCING SUBSTANCES OF HUMAN BLOOD IN PATHOLOGICAL CONDITIONS.

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In earlier papers [Herbert, Bourne and Groen, 1930; Herbert and Bourne, 1930] it was shown that there is in the blood corpuscles non-diffusible reducing material, which is responsible for the discrepancies observed when blood-sugar is determined by different methods. This material passes into Folin-Wu filtrates, and raises the apparent sugar content when these filtrates are analysed by the Hagedorn-Jensen, Shaffer-Hartmann and Folin-Wu methods; the Benedict [1928] method alone gives true sugar figures for Folin-Wu filtrates. The non-sugar material is absent from zinc hydroxide filtrates made by Somogyi's technique [1929] and from the tungstic acid filtrates from non-haemolysed blood described by Herbert and Bourne [1930]; all the analytical methods agree when applied to these two filtrates. Folin [1930] has also described a method of precipitating non-haemolysed blood with tungstic acid, essentially the same as ours, and recommends this instead of the Folin-Wu method for the Folin-Wu system of blood analysis, and especially for estimations of uric acid.

The effects produced by the non-diffusible reducing substance are consistent with the view that it is glutathione. If normal whole blood contains 40–60 mg. of reduced glutathione per 100 cc. (all present in the corpuscles) as is suggested by estimations based on the nitroprusside reaction, then the reducing effects in the different methods are completely explained.

In the previous work only normal human blood was used. It was thought that further estimations of the non-diffusible reducing substance of corpuscles, in pathological conditions, might throw some light on the physiological function of glutathione. The non-diffusible reducing substance has been estimated by taking the difference between the figures given by tungstic acid filtrates from haemolysed and non-haemolysed blood, analysed by the ferricyanide method. In normal blood this non-diffusible reducing substance accounts for practically the whole of the non-sugar reducing substance; in pathological conditions this may or may not be so, but our method of estimation gives figures for the non-diffusible substance which will be valid whether there is any diffusible non-sugar reducing substance or not.

[1] Holding a grant from the Medical Research Council.

Since the non-diffusible reducing substance occurs in corpuscles, it was to be expected that its concentration in whole blood would depend on the corpuscle content of the blood. The figures for non-diffusible material must be considered in relation to the haematocrit readings. The present series of cases is short, but it includes examples of polycythaemia and severe anaemia; haematocrit readings for corpuscle volume range from 12 % to 67 %.

Methods.

Oxalated venous blood was used. The samples were precipitated by each of three methods: (1) the Folin-Wu technique [1920]; (2) Somogyi's zinc hydroxide technique [1929], and (3) the tungstic acid precipitation of non-haemolysed blood [Herbert and Bourne, 1930], which is indicated in the tables as "sulphate-tungstic filtrate." All determinations were made by the Hagedorn-Jensen method, because in this method the non-diffusible material exerts a maximum effect on the apparent sugar figure. In all three methods of precipitation the dilution of the blood was 1 in 10, and in each case 1 cc. of filtrate was taken and diluted with about 11 cc. of water; 2 cc. of Hagedorn-Jensen ferricyanide solution were added, and the remainder of the analysis was carried out as in the Hagedorn-Jensen method. The difference between the figures for the Folin-Wu filtrate and the "sulphate-tungstic" filtrate represents the non-diffusible reducing substance.

It was not always possible to work on freshly-drawn blood, and many of the samples had stood for 1–3 hours after drawing, and may have undergone slight glycolysis. The loss of glucose was of no importance, as we were concerned with the non-sugar fraction, but it was desirable to determine whether this underwent any change on standing at room temperature, and a few experiments were made on this point, and are recorded in Table I. There appears to be some slight fluctuation in the non-sugar fraction, apart from experimental error, but the variations likely to occur in 3 hours are too small to affect the main significance of our results.

Results.

In Table II, the blood samples are arranged in ascending order of the haematocrit readings for corpuscle volume. In general the samples with low corpuscle content show low values for the non-sugar material in whole blood, whereas the polycythaemic blood samples show high figures. Case 23 is striking, as it shows a true sugar figure of 41 mg./100 cc. associated with a non-sugar figure of 70 mg./100 cc.

The next question is whether there is any significant variation in the concentration of non-diffusible reducing substance per unit corpuscle volume. In Table II the calculated figures for non-diffusible reducing substance, as glucose per 100 cc. corpuscles, are given. These figures may be misleading, as the experimental error is greatly magnified by the calculation when the haematocrit

Table I. *Changes in glucose, and non-diffusible substance in blood allowed to stand at room temperature.*

Case No.	Hours after drawing	Reducing substance, mg./100 cc. whole blood, expressed as glucose in: Folin Wu filtrate (*a*)	Sulphate-tungstic filtrate (*b*)	Zinc filtrate (*c*)	Non-diffusible fraction (*a*–*b*)
8	0	125	107	110	18
(chronic nephritis)	3	123	97	100	26
	6	113	93	92	20
4	3	144	131	132	13
(neoplasm of liver)	5	141	—	125	16 (*a*–*c*)
10	0	138	108	110	30
(normal)	½	132	102	107	30
	1	129	99	99	30
	2	128	101	101	27
	3	131	95	99	36
	23	65	36	—	29
	72	32	12	10	20
16	0	118	93	95	25
(myeloid leucaemia)	1	105	75	—	30
	6	69	34	—	35
	26	34	8	—	26

Table II.

Case No.	Age yrs.	Diagnosis	Total red corpuscles per mm.³	Haemoglobin % of normal (Haldane scale)	Reducing substance as glucose mg./100 cc. (*a*) Folin-Wu filtrate	(*b*) Sulphate-tungstic filtrate	(*c*) Zinc filtrate	Non-diffusible fraction (*a*–*b*)	Corpuscle volume % of whole blood	Non-diffusible fraction as glucose mg./100 cc. corpuscles
1	1 3/12	Haemolytic anaemia	2,760,000	20	116	102	101	14	12	117
2	52	Pernicious ,,	1,310,000	48	115	97	97	18	18	100
1	1 3/12	Haemolytic ,,	4,210,000	48	81	60	—	21	22	95
3	25	Haemorrhage (ectopic gestation)	—	—	138	124	125	14	22	64
4	65	Neoplasm of liver	2,070,000	42	144	131	132	13	23	57
5	—	Post-partum haemorrhage	2,560,000	59	147	125	127	22	24	92
6	58	Haematemesis (oesophageal varix)	3,170,000	59	124	103	103	21	26	81
7	—	Acute nephritis. Uraemia	3,280,000	66	98	74	72	24	26	92
8	64	Chronic nephritis. Uraemia	—	—	123	97	100	26	26	100
9	66	Osteoarthritis	—	—	143	115	112	28	31	90
10	72	Sulphaemoglobinaemia	—	—	151	132	131	19	33	58
11	25	Acute yellow atrophy of liver	—	—	129	102	108	27	37	73
12	35	Renal carcinoma	—	—	117	84	84	33	41	80
13	9	Malformed heart	6,170,000	95	129	101	101	28	43	65
14	31	Normal	—	—	129	99	99	30	43	70
15	5½	Staphylococcal septicaemia	—	—	188	164	163	24	44	55
16	31	Sulphaemoglobinaemia	—	—	105	81	80	24	44	55
17	61	Gout	—	—	145	109	108	36	44	82
18	63	Carcinoma of colon	5,070,000	103	128	88	81	40	46	87
19	—	Gout	—	—	109	80	78	29	48	60
20	29	Malformed heart	7,180,000	124	118	70	69	48	57	84
21	12	,,	—	164	125	86	86	39	62	63
22	22	,,	9,700,000	146	154	88	97	66	67	99
23	5/12	,,	8,400,000	140	111	41	41	70	67	104

Case 1. Severe haemolytic anaemia; no jaundice, but increased fragility of corpuscles and urobilinuria. Case 7. Acute nephritis; blood urea 103 mg./100 cc. Case 8. Chronic nephritis with high blood pressure. Blood urea on the day of the test, 49 mg./100 cc. Previous figures for blood urea ranged from 56 to 152 mg./100 cc. Case 9. Osteoarthritis. Blood uric acid 4·2 mg./100 cc. Cases 10 and 16. Sulphaemoglobinaemia due to methylacetanilide. Case 17. Gout. Blood uric acid 3·3 mg. per 100 cc.

reading is low, whereas in cases with normal or raised corpuscle content the calculation has proportionately much less effect.

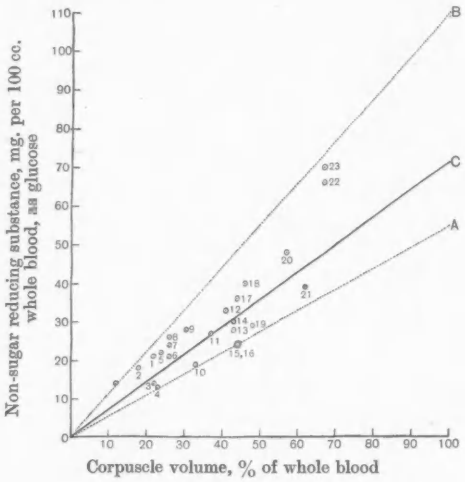

Fig. 1.

The graph, Fig. 1, has been devised to present the results in their true perspective and to show their relation to the normal. The observed corpuscle volumes are plotted as abscissae and the observed figures for non-diffusible reducing substance (as mg. glucose per 100 cc. whole blood) are plotted as ordinates. The numbers at the points correspond to the case numbers in Table II. On the basis of our previous findings we may take the average normal figure for the non-diffusible reducing substance as 29 mg./100 cc. whole blood, and the average normal corpuscle volume as 41 %. This indicates that the normal concentration of non-diffusible reducing substance, expressed as glucose, is 71 mg./100 cc. corpuscles. The point *C* represents this value as determined on pure corpuscles, and the line *OC* is the locus of results for samples of whole blood which contain this same concentration in corpuscles, but in which the proportion of corpuscles to plasma varies. *OA* and *OB* are constructed in the same way, and are the loci of results corresponding to 55 and 110 mg. per 100 cc. corpuscles, respectively. These are the minimum and maximum concentrations in corpuscles, found in the present series of results. If the concentration of non-diffusible reducing substance per 100 cc. corpuscles were constant, all the points would fall on the line *OC*. They do not, but in most cases one cannot say whether the divergence is significant. For example, in Case 1 the haematocrit reading was 12 and the non-diffusible substance, mg./100 cc. whole blood, was 14. This brings the plotted result just above the line *OB*, but a result of 8·5 instead of 14 would have brought it to the line *OC*, and this variation might be due to experimental error.

If we take cases 1 to 21 we see that the divergence from the line *OC* is ±7 mg./100 cc. whole blood, and of these 21 cases, only 5 cases diverge by more than ±5 mg./100 cc. Such variations can scarcely be regarded as significant, since the figures for non-diffusible reducing substance are obtained by taking the difference between two analytical results, and are therefore affected by the experimental error of both. Cases 22 and 23 show a very definite divergence from the normal average. Both had a haematocrit reading of 67 %, and the figures for non-diffusible substance were 66 and 70 mg./100 cc. whole blood. The point on *OC* corresponding to a haematocrit reading of 67 % is 48 mg. non-diffusible substance per 100 cc. whole blood. The divergence here cannot be experimental. Both were cases of marked secondary polycythaemia due to malformation of the heart. Unfortunately we have not sufficient data to fix the normal range, but these cases are definitely above our normal average, and they are the only cases in which there is a really significant deviation.

It would be of interest to obtain further figures, to fix the normal range, and to investigate primary and secondary polycythaemias. More accurate results in anaemic cases could be obtained by working with pure corpuscles. The only definite result that emerges from the present series of observations is that the non-diffusible reducing substance of whole blood varies with the corpuscle content, and that variations in this substance have no significance when considered apart from corresponding variations in corpuscle volume.

Throughout the series there is excellent agreement between the figures given by the sulphate-tungstic filtrate and those given by Somogyi's zinc filtrate. This suggests that the non-sugar material in these samples is of the same nature as that in normal blood. It shows, at least, that there is no non-diffusible reducing substance which escapes precipitation by zinc hydroxide, and conversely, that there is no diffusible reducing substance which is precipitated by zinc hydroxide.

It is probable that the non-diffusible material is glutathione. The results may be expressed in terms of glutathione by multiplying the figures, expressed as glucose, by 1·8[1] [Herbert, Bourne and Groen, 1930]. The range of variation for the series is therefore 99 to 198 mg. per 100 cc. corpuscles, as reduced glutathione. These figures must not be regarded as equivalent to glutathione estimations, as it is possible that other non-diffusible reducing substances, precipitable by zinc, may occur in pathological conditions.

One case of myelogenous leucaemia has been examined and the results are given in Table III. In the earliest sample the volume of leucocytes was a considerable proportion of the total cell volume. When the leucocyte count was 400,000 to 500,000 per mm.^{3}, it appeared that about half the corpuscle deposit consisted of leucocytes, though we found it impracticable to estimate the red cell volume and white cell volume separately. Later, when the leucocytes fell to 20,000 per mm.^{3}, the leucocyte volume was only a small proportion of the total. In spite of these marked variations in the proportions of red

[1] This factor applies only to results obtained by the ferricyanide method.

Table III.

Case 24. J. C., aged 15. Myelogenous leukaemia.

Date	Total red corpuscles per mm.³	Total leucocytes per mm.³	Corpuscle volume	Reducing substance expressed as glucose mg./100 cc. Folin-Wu filtrate	Sulphate-tungstic filtrate	Zinc filtrate	Non-diffusible reducing substance: As glucose mg./100 cc. whole blood	As glucose mg./100 cc. corpuscles	As reduced glutathione mg./100 cc. corpuscles
10. ii. 30	—	408,000	—	—	—	—	—	—	—
28. ii. 30	—	—	38	72	34	34	38	100	180
3. iii. 30	—	510,000	—	—	—	—	—	—	—
15. iii. 30	—	191,000	—	—	—	—	—	—	—
24. iii. 30	—	144,000	—	—	—	—	—	—	—
25. iii. 30	2,420,000	110,000	29	100	70	70	30	103	182
31. iii. 30	—	111,000	—	—	—	—	—	—	—
4. iv. 30	—	53,000	—	—	—	—	—	—	—
16. v. 30	2,260,000	20,000	25	118	93	95	25	100	180

and white cells, the non-sugar substance (expressed as glucose or glutathione mg. per 100 cc. corpuscles) remained very constant. This suggests that the concentration of non-diffusible reducing substance is the same in leucocytes as in red cells, so that the same figure is obtained whatever the constitution of the corpuscle mass. If so, the non-diffusible substance from leucocytes is liberated in the Folin-Wu precipitation and not in the sulphate-tungstic method. This is curious, as one would not expect the leucocytes to be broken up more readily in the one than the other. Further investigation is required.

From the present series of analyses it is clear that in all work on the non-sugar reducing material it is necessary to consider the corpuscle volume, and to express concentration per 100 cc. of corpuscles rather than per 100 cc. of whole blood. A rise in the non-sugar reducing substance has been noted by Sure and Smith [1929] in animals suffering from severe vitamin B-deficiency, but these animals were dehydrated at the time of the observation.

From the standpoint of routine sugar analysis, it should be remembered that if any method affected by the non-sugar material is used, the errors will be greater in cases with anhydraemia or polycythaemia than in those in which the corpuscle volume is normal.

Summary.

The non-diffusible reducing substance has been estimated in a series of pathological bloods, and its concentration in whole blood has been shown to vary with the haematocrit reading for the corpuscle volume. This was to be expected, since the non-sugar substance is a constituent of corpuscles, most probably glutathione.

The results in one case of myelogenous leucaemia lead to the suggestion that the concentration of non-diffusible reducing substance in leucocytes and erythrocytes is of the same order.

The concentration of the non-diffusible reducing substance in corpuscles varies, but in the majority of cases the variation from the normal average is within experimental error. In two cases of marked secondary polycythaemia the non-diffusible substance is above the normal average.

We wish to express our thanks to members of the staffs of St Bartholomew's Hospital and the Belgrave Hospital for Children, for giving us opportunities to make the observations here recorded.

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CCII. THE BIOLOGICAL VALUES OF PROTEINS. II. THE BIOLOGICAL VALUE OF PURIFIED CASEINOGEN AND THE INFLUENCE OF VITAMIN B_2 UPON BIOLOGICAL VALUES, DETERMINED BY THE BALANCE SHEET METHOD.

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In 1909 Thomas introduced the term biological value of protein to express the relative value of different proteins for compensating the daily nitrogenous loss, and defined this as the number of parts of body nitrogen replaceable by 100 parts of the nitrogen of the foodstuff [1909]. He suggested three formulae, differing as regards the treatment of the nitrogen excreted in the faeces, by which to calculate this value from the data of experiments on the nitrogenous exchange. Since that time determinations of the biological values of many proteins have been made by various observers (McCollum [1911, 1914] on the pig; Martin and Robison [1922] on man; Mitchell and his co-workers [1924, 1926, 1927] on the rat; Wagner [1923] on children; and others). The results of these workers show a wide range of variation. For instance, the biological value of the proteins of cow's milk has been variously assessed as 51, 74, 85, 93 and 100 and that of oat protein as 47, 65 and 79.

The provision of adequate amounts of vitamin B in the experimental diet presented serious difficulties to the earlier workers on the subject. Since all the known rich sources of this vitamin were substances rich in nitrogen, as, for example yeast, investigators were faced with the alternatives either of giving a diet deficient in vitamin B, or of adding to the diet appreciable amounts of nitrogen of unknown biological value, and thus decreasing the accuracy of their results. In many experiments the only source of the water-soluble B vitamin in the diet was the protein under investigation. Some proteins would contain considerable amounts of this factor while from others it would be almost entirely absent. In the latter case difficulty would be experienced in maintaining appetite sufficient to prevent the intake of calories falling below the required minimum. A further complication came to light in recent years with the discovery that there are at least two water-soluble B vitamins, having different functions in the body and, in some cases, a different distribution in nature. Mitchell and Carman [1924] attacked the problem of the supply of these vitamins by giving the rats which formed the subject of

their experiments, a daily dose of yeast vitamin-Harris powder (a preparation made from yeast and containing both the B vitamins). The daily ration contained 2·3 mg. of nitrogen.

The work of Kinnersley and Peters [1925, 1927] and of Chick and Roscoe [1927, 1928] has shown that concentrates of these vitamins can be prepared from yeast, with a nitrogen content considerably lower than that of the yeast vitamin-Harris powder. Hence arose the decision to attempt a redetermination of the biological values of proteins by means of metabolism experiments in which the use of such preparations would ensure an adequate supply of both the B vitamins. Rats were selected as the experimental animals. Mitchell has elaborated and tested a technique for use with these animals and has shown that consistent results can be obtained. The method adopted here, however, was that used by Korenchevsky (unpublished results) and Chick and Roscoe [1930].

Purified caseinogen was the first protein selected. There are not many previous determinations of the biological value of this protein. Michaud [1909] fed a dog on a daily ration of caseinogen equivalent to its total daily output of nitrogen on a nitrogen-free diet and obtained a negative balance. Thomas [1909, 1910] gave caseinogen a value of 70 as the result of experiments on an adult human being. McCollum in experiments on pigs in which positive balances of nitrogen were recorded, obtained a value for caseinogen of 67, while the figure given by Mitchell [1924, 2, 4] as the result of experiments on rats was 71 and 75. Martin and Robison [1922], however, gave whole milk proteins a value of only 51.

Since the purified caseinogen (see below) used in this laboratory is devoid of the B vitamins, experiments to determine the biological value of this protein offered a good opportunity to investigate the influence of vitamins B_1 and B_2 on nitrogen metabolism. Previous work on this subject by Karr [1920] was carried out before the dual nature of vitamin B was known. A dog, fed on a diet containing a large amount of caseinogen but no source of vitamin B, maintained a small positive balance of nitrogen for some weeks. When yeast was added to the diet the retention of nitrogen was increased. In similar experiments on two other dogs the ingestion of yeast changed a negative balance of nitrogen into a positive one. Karr attributed these results to the biological value of the yeast protein rather than to the vitamin content of the yeast.

Studies on the distribution of vitamin B_2 have shown it to be usually associated with proteins of good biological value such as meat, milk and eggs, rather than with those of low biological value, such as cereals and pulses [Aykroyd and Roscoe, 1929]. This suggested the possibility that the presence of vitamin B_2 might be essential to secure an economical use of ingested nitrogen. Discrepancies between the results obtained by different investigators might be due to differences in the vitamin B_2 content of their diets, and it was conceivable that differences in the biological values of proteins might ulti-

mately prove to be related, in many cases, to variations in vitamin B_2 content rather than to essential differences in protein constitution.

An investigation was therefore planned in which the biological value of purified caseinogen at different levels of intake would be determined in the presence and absence of vitamin B_2.

Experimental.

General plan of experiments.

Two series of experiments were carried out on adult male rats of 350–450 g. wt. In Series 1 rats 1, 2, 3 and 4 were used, and in Series 2 rats 2, 3, 5, 6, 8 and 9. Each experiment involved one pair of rats treated as a single unit in one metabolism cage. The basal nitrogen expenditure was first determined using the "nitrogen-free" diets described below. In the succeeding experiments the same diets were used with added caseinogen in proportions varying from 4 to 16 % of the total dry diet. In half the experiments of each series a daily ration of a watery yeast extract (fraction C [Chick and Roscoe, 1929]), which contains both members of the water-soluble B complex, was administered; in half, Peters's antineuritic concentrate prepared from yeast [Kinnersley and Peters, 1925, 1927], containing vitamin B_1 only, was given. The experiments lasted from 4 to 5 days except in the case of those on the nitrogen-free diet, where, owing to the greater tendency to lose weight, it was thought wiser to terminate them on the third day. In all cases there was a pre-experimental period of 2 days in which the experimental diet and dose were ingested but no analyses were made. Between each experiment in Series 2 the rats were allowed to "rest" for at least 7 days during which time they received a diet containing 16 % of "light white" caseinogen. On each day except the last they also received 1 g. of dried yeast. In Series 1 the length of the "rest" depended on the nature of the preceding experiment.

Materials used.

The caseinogen was prepared from "light white casein" (British Drug Houses), a sodium caseinogenate manufactured by the method of Hammarsten. It was purified by re-precipitation with acetic acid and frequent washing by decantation with 0·05 % acetic acid. It was then extracted with dilute acidified alcohol for 96 hours in a Soxhlet apparatus, dried at a low temperature and finally roasted for 3 days at 120°. Caseinogen prepared by this method contains no B vitamins [Chick and Roscoe, 1928]. The nitrogen content on the dry weight was 14·81 %.

The fraction C, prepared from yeast extract [Chick and Roscoe, 1929] had a sp. gr. of 1·158 and a nitrogen content of 0·278 % by volume. The daily ration of 0·5 cc. per rat contained 1·4 mg. of nitrogen.

The sample of Peters's vitamin B_1 concentrate from yeast had a sp. gr.

of 1·049 and a nitrogen content of 0·633 % by volume. Each rat received 4 drops daily containing 0·7 mg. of nitrogen.

I am indebted to Dr Chick and Miss Roscoe for supplying me with these materials.

Diets. The "nitrogen-free" diets used in Series 1 and 2 respectively were constituted as follows.

Nitrogen-free diet. Series 1:

Corn starch	735 g.
Sugar	60
"Hardened" arachis oil	130
Cod-liver oil	20
Salt mixture	50
Calcium carbonate	8

This diet contained 0·0297 g. nitrogen and 500 kg. Calories per 100 g. of dry weight. As this diet was not well taken in all cases, certain modifications were introduced to make it more palatable. These consisted of an increase in the proportion of sugar and of the substitution of beef dripping for the arachis oil. The modified diet had the following constitution:

Nitrogen-free diet. Series 2:

Corn starch	735 g.
Sugar	90
Clarified beef dripping	100
Cod-liver oil	20
Salt mixture	50
Calcium carbonate	8

This diet contained 0·0279 g. of nitrogen and 380 kg. Calories per 100 g. of dry weight.

Technique of the metabolism experiments.

The main features of the technique are described in the preceding paper [Chick and Roscoe, 1930]. In Series 2 an improvement was introduced into the method of measuring the nitrogen intake. The procedure of estimating the nitrogen content in the basal diet after mixing the caseinogen with the other constituents of the diet was abandoned owing to the difficulty of ensuring a sufficiently even mixture for correct sampling. The following procedure was substituted. A large quantity of the nitrogen-free diet was prepared without the addition of water, and samples were taken for analysis. This air-dry diet was kept in a tin in the refrigerator room, the diet bottle for each experiment being filled from this store. The purified caseinogen was dried at 110° for 24 hours and transferred to a heated weighing bottle. After cooling in a desiccator this was weighed. When the daily ration of the nitrogen-free food was weighed out from the diet bottle into the daily ration pots, the approximate amount of caseinogen needed to give the required protein content

to the diet was added from the weighing bottle. The diet and the caseinogen were well mixed in the daily ration pots with enough water to give a liquid paste. At the end of the experiment the weighing bottle, containing the unused caseinogen, was heated, cooled and weighed as before, the difference between the two weighings giving an accurate measure of the amount of caseinogen removed. The diet bottle was also weighed before and after the experiment to arrive at the amount of basal diet consumed. Any residue remaining in the daily ration pots was collected and analysed for nitrogen, in order to make allowance for the unconsumed nitrogen. After experience of the animal's appetite it could be usually arranged that this amount was small and insignificant.

Calculation of the biological value of a protein.

Martin and Robison [1922] and Mitchell [1924, 1, 3] have discussed at length the various problems involved in the calculation of the biological value of a protein from the data provided by experiments in which the nitrogen balance is determined. The method of calculation used here is based on the work of these observers, their methods being in turn based on the formulae of Thomas [1909, 1910].

Martin and Robison showed that the situation could be expressed on a graph (Fig. 1) in which the abscissae represent real nitrogen intake (x) and

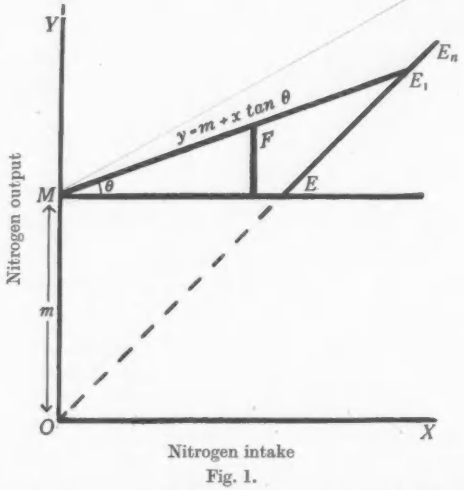

Fig. 1.

the ordinates real nitrogen output (y); m ($= OM$) is the output on a nitrogen-free diet. The formula used here for calculation of biological values is based on that of Mitchell but has two modifications. To express output, the "true" output (*i.e.* urine nitrogen + endogenous faecal nitrogen) is used instead of urine nitrogen alone, as it is thought that the endogenous faecal nitrogen should be regarded as forming part of the basal nitrogen expenditure. Secondly no

correction is made in the estimation of the endogenous urine nitrogen to allow for the variations in body weight of the rats, as such a correction is not applicable to experiments with adult rats where variations in body weight will be related to alterations in the amount of body fat and will not therefore be associated with variations in basal nitrogen expenditure.

The formula of Mitchell,

$$\text{B.V.} = 100 \times \frac{\text{nitrogen retained}}{\text{nitrogen absorbed}},$$

is therefore modified thus:

$$\text{B.V.} = 100 \times \frac{\text{true intake} - (\text{true output} - \text{output in nitrogen-free experiment})}{\text{true intake}}.$$

From the graph of Martin and Robison (Fig. 1) this becomes:

$$\text{B.V.} = 100\frac{x-(y-m)}{x}.$$

Since from the graph $y = m + x \tan \theta$,

$$\text{B.V.} = 100\frac{x-(m+x\tan\theta-m)}{x} = 100\,(1 - \tan\theta).$$

This is the same equation as that deduced by Martin and Robison from Thomas's formula B.

The method of calculating the biological values of proteins used in these experiments was, therefore, as follows.

From the faecal nitrogen of the nitrogen-free experiments, the endogenous faecal nitrogen per 100 g. of dry food ingested was calculated for each set of rats [see Mitchell, 1924, 1]. This figure was used in each experiment to determine the endogenous and exogenous fractions of the total faecal output. The endogenous faecal nitrogen was added to the total urinary nitrogen to obtain the figure for the true output, while the exogenous portion, representing unabsorbed food nitrogen was subtracted from the total nitrogen ingested to obtain the true intake. The nitrogen of the yeast concentrates was, contrary to the procedure of Mitchell, included in the total nitrogen intake. The figures for true intake and true output were used to plot the point F on the graph, m having been already plotted from the intake and output in the nitrogen-free experiment. Tan θ was measured and the biological value calculated from the equation B.V. $= 100\,(1 - \tan\theta)$.

Discussion of experimental results.

In those experiments in which vitamin B_1 only was supplied considerable difficulty was experienced in maintaining the intake at a sufficiently high level, for the rats showed a marked loss of appetite within 48 hours of deprivation of vitamin B_2. The rapidity with which this loss of appetite appeared is consistent with the inability of the rat to store large reserves of this vitamin [Chick and Roscoe, 1927]. When the total calorie intake falls below a certain figure, the animal will use some of the protein of the diet to supply energy rather than to replace body nitrogen. Under these circumstances the bio-

Table I. *Details of the experiments in which an adequate intake of calories from fat and carbohydrate combined was secured. The figures given represent average daily values for one rat.*

Exp. No.	Rats No.	Date	B vitamins given	Amount of protein in diet % of dry weight	Non-protein intake of calories per kg. of body weight Cals.	Average body weight g.	Change in body weight, as % of initial body weight	Intake of nitrogen		Faecal nitrogen			Nitrogen in urine mg.	Output of nitrogen		Balance of nitrogen mg.	Biological value $100 \times (1 - \tan \theta)$ using true output and true intake	Biological value $100 \times (1 - \tan \theta)$ using urine nitrogen and true intake
								Total mg.	Total mg.	Total mg.	Endogenous mg.	Exogenous mg.		Total mg.	True mg.			
Series 1:		1929																
1_3	2 and 4	May 22–25	B_1 and B_2	0·2 "N-free"	150	310	−1·3	4	4	17	17	—	65	82	82	−78	—	—
9_3	1 and 3	Aug. 20–24	B_1 and B_2	5·6	125	294	−0·8	75	74	19	18	1	101	120	119	−45	**49**	49
9_5	2 and 4	,, 20–24	B_1	5·6	130	320	−1·5	84	78	23	17	6	106	129	123	−44	**45**	45
7_3	2 and 4	,, 13–17	B_1 and B_2	4·4	170	331	−0·4	90	87	26	23	3	108	134	131	−44	**41**	48
4_3	1 and 3	July 1–5	B_1 and B_2	7·7	140	294	−0·5	122	116	27	21	6	124	151	145	−29	**44**	43
6_5	2 and 4	July 29–Aug. 2	B_1 and B_2	15·4	142	299	+5·0	278	260	38	20	18	204	242	224	+36	**45**	45
5_3	1 and 3	July 23–27	B_1 and B_2	15·4	145	294	+6·0	282	274	32	24	8	196	228	220	+54	**49**	51
Series 2:																		
10	2, 3, 5, 6, 8 and 9	Nov. 18–21	B_1 and B_2	0·2 "N-free"	185	402	−4·0	7	7	21	21	—	71	92	92	−85	—	—
11_2	5 and 8	Dec. 1–7	B_1	5·6	130	417	−4·0	155	113	65	23	42	128	193	151	−38	**45**	47
11_3	3 and 6	,, 1–7	B_1	5·6	130	415	−2·0	160	126	57	23	34	133	190	156	−30	**47**	48
11_7	2 and 9	,, 1–6	B_1 and B_2	5·6	150	425	−1·0	190	153	62	25	37	145	207	170	−17	**46**	49
12_3	3 and 6	,, 15–20	B_1 and B_2	7·2	135	413	−3·0	206	160	70	24	46	144	214	168	− 8	**51**	52
8_{3a}	5 and 8	Aug. 26–29	B_1 and B_2	12·9	130	413	+2·0	278	255	41	17	24	214	255	231	+24	**43**	44
8_3	5 and 8	,, 19–23	B_1 and B_2	12·9	145	410	−1·0	304	284	38	18	20	240	278	258	+26	**40**	39
																Mean biological value	**45**	47

logical value will be underestimated. The experiments described here indicate that the use of protein for fuel is not significant if the calorie intake from fat and carbohydrate combined is greater than about 125 kg. Calories per day per kg. of rat. (The calculations of calorie intake are only approximate.) All experiments in which it falls below this figure have, therefore, been rejected as unreliable for calculating the biological value of the protein.

Table I gives details of six experiments in Series 1 and six experiments in Series 2 in which the calorie intake was regarded as satisfactory. The figures for the experiments with nitrogen-free diets are given at the head of each Series. In Series 1 the experiment with a nitrogen-free diet on rats 2 and 4 is not used in the calculation, as vitamin B_1 only was given. As a result of loss of appetite, the calorie intake became too low and the loss of body weight too high for the figures to be trustworthy. As the two sets of rats behaved similarly in other experiments it was thought best to calculate the biological value in the experiments on rats 2 and 4 from the values obtained in the experiment with a nitrogen-free diet on rats 1 and 3.

In Series 2 biological values are calculated using the average figures derived from the nitrogen-free experiments on the three pairs of rats, as the individual figures obtained from the experiment with nitrogen-free food on rats 2 and 9 and that on rats 3, 5, 6 and 8, were almost identical.

In the last column but one of Table I are given the biological values of purified caseinogen as calculated from the results of each experiment. These range from 40 to 51 with a mean value of 45. Four experiments are included in which a positive nitrogen balance was obtained.

In Table II are collected all the experiments rejected on account of low

Table II. *Some details of the experiments rejected on account of insufficient calorie intake.*

Exp. No.	Rats No.	Date	B vitamins given	Amount of protein in diet % of dry wt.	Non-protein intake of calories per kg. of body weight Cals. per day	Average body weight g.	Change in body weight = % of initial body weight per day	Biological value $100\times(1-\tan\theta)$ using true output and true intake
Series 1:		1929						
1_3	2 and 4	May 22–25	B_1 and B_2	0·2 "N-free"	150	310	−1·3	—
7_3	1 and 3	Aug. 13–17	B_1	4·4	100	302	−1·2	29
4_3	2 and 4	July 1–5	B_1	7·7	120	309	−1·1	29
5_3	2 and 4	,, 23–27	B_1	15·4	80	310	−1·3	27
6_3	1 and 3	July 29–Aug. 2	B_1 and B_2	15·4	105	293	−0·5	42
Series 2:								
10	2, 3, 5, 6, 8 and 9	Nov. 18–21	B_1 and B_2	0·2 "N-free"	185	402	−4·0	—
12_2	5 and 8	Dec. 15–20	B_1	6·5	105	407	−1·3	40
12_7	2 and 9	,, 15–20	B_1 and B_2	6·7	115	419	−0·9	37
		1930						
13_2	3 and 6	Jan. 12–17	B_1	11·3	75	426	−0·8	31
13_2	5 and 8	,, 12–17	B_1 and B_2	12·4	100	408	−0·4	36

calorie intake. It will be seen that, as was anticipated, these have, with two exceptions, 12_2 and 6_3, biological values of less than 40.

In the last column of Table I are shown the biological values obtained when the calculations are made using urine nitrogen in place of true output, as is done by Mitchell. It will be seen that the values obtained by this method do not show significant differences from those obtained by the other method, the mean values being respectively 47 and 45.

The influence of vitamin B_2 on the biological values of proteins.

An examination of these results shows that vitamin B_2 does not itself exert an influence on the economical use of ingested nitrogen. In Table I there are three experiments in which the animals received no vitamin B_2, but the estimates of biological value (45, 47 and 46) are not lower than those of the experiments in which both vitamins were supplied. In Table II, on the other hand, there are three experiments in which, in spite of the ingestion of both B vitamins, the calorie intake was low, and in these the biological values were also low.

It was originally intended to investigate the influence of vitamin B_1 on nitrogen metabolism as well as that of vitamin B_2, but so rapid is the decline in appetite when the first factor is removed from the diet that it has not so far been possible to secure a sufficiently high intake to give trustworthy results.

Biological values at different levels of intake.

Martin and Robison [1922] questioned on theoretical grounds the assumption of Thomas that the biological value of a protein would be a constant for different levels of protein intake. If it were not constant, ME_1 in their graph (Fig. 1) would be curved. They found, however, that in the case of wheat protein ME_1 did indeed appear to be a straight line. The results obtained in this paper give no evidence of a variation of biological value with variations in the level of protein intake when caseinogen is used. The validity of the equation B.V. $= 100\,(1 - \tan\theta)$ is based on the assumption that ME_1 is a straight line. Confirmation of this is given by the fact that the biological values calculated by means of this equation for levels of caseinogen intake varying from 4 to 16 % of the diet show no wide range of variation.

Mitchell [1924, 2] determined the biological values of a number of proteins when the intake represented 5 % and 10 % of the diet, respectively. With the higher percentage of protein in the diet, though there might not be increased intake of nitrogen, the values obtained were consistently lower. He attributed this in part only to variations in biological value at different levels of intake. Since in his first set of experiments the animals were stationary in weight, while in the second they were growing, he also suggested that the biological value of a protein for growth was different from the value when maintenance only was required. The experiments described in this paper do

not support this theory. In those experiments in which the rats were increasing in weight the average biological value was 46, while the average figure for the entire series was 45. It is to be noted, however, that the rats used in these experiments were slowly-growing adults, whereas those used by Mitchell were young and growing rapidly.

The biological value of 46 for caseinogen is much lower than those determined by McCollum and Mitchell, 67 and 71 respectively. This may be due to differences in technique, or possibly to a higher purity in the sample of caseinogen used. Contamination with lactalbumin would, of course, increase the apparent biological value of the caseinogen.

SUMMARY.

1. The biological value of purified caseinogen has been redetermined by means of 12 metabolism experiments on adult male rats. In these experiments the B vitamins were supplied by means of concentrates containing only small amounts of nitrogen—an improvement on the technique previously available.

2. The formula used here to calculate the biological value of a protein, B.V. $= 100\ (1 - \tan \theta)$, is based on the work of Thomas, Martin and Robison, and Mitchell.

3. The biological value of purified caseinogen was found to be 45, a figure considerably lower than that found by previous workers.

4. In experiments, in which the intake of calories from fat and carbohydrate combined fell below a certain value, the use of ingested protein for fuel is at once indicated by the underestimation of the biological value.

5. The absence of either vitamin B_1 or B_2 from the diet caused a decline in appetite within 48 hours. So great was the decline in appetite in the absence of vitamin B_1 that the intake of calories was too low to allow reliable figures to be obtained for the calculation of biological values.

6. The absence of vitamin B_2 from the diet does not appear to prevent the economical use of ingested nitrogen, provided the calorie intake from fat and carbohydrate is adequate.

7. There is no evidence in these experiments either of variations in biological value at different levels of intake or of the existence of different biological values for growth and maintenance.

I wish to express my gratitude to Sir Charles Martin for his advice and criticism and to Dr H. Chick for suggesting the problem to me and for her constant help and encouragement throughout the investigation.

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CCIII. A COMPARISON OF THE COLORIMETRIC AND ELECTROMETRIC METHODS FOR THE DETERMINATION OF THE p_H OF SERUM OR PLASMA.

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The colorimetric method, because of its simplicity and the rapidity with which determinations may be made, has been the object of many biochemical investigations. Levy, Rowntree and Marriott [1915] devised the dialysis method in which phenol red was added to the dialysate of the blood, and the resulting colour compared to a set of standards. Later, Dale and Evans [1920–21] improved this method by preventing the loss of CO_2 from the system. Cullen [1922] eliminated the error due to the Donnan effect in the dialysis method by a procedure employing diluted plasma, in which an empirical correction, determined by a comparison of the colorimetric results obtained at 20° with those obtained by the hydrogen electrode at 38°, allowed for protein, salt, dilution, and temperature effects. Employing the Gillespie principle, Hastings and Sendroy [1924] developed a system of bicolour standards which permitted the colorimetric determination of the p_H of plasma or of whole blood at 38°, obviating the use of the empirical Cullen C correction.

The accuracy of the colorimetric method.

The value of the Cullen C correction for human plasma is 0·22 p_H, thus the calculation for the reaction of blood at 38° is:

$$[p_H]_{38°} = [p_H]_{20°} - 0{\cdot}22.$$

Bennett [1926] apparently found that the value of C was not a constant for the same blood under various conditions. Hastings and Sendroy [1924], on the other hand, reported that the results of the colorimetric and electrometric methods on different species of blood agreed very well when the colorimetric determination was carried out at 38°. In addition to their own procedure, they employed the single colour standard method of Cullen, and the micro-modification of Cullen's method devised by Hawkins [1923].

Austin, Stadie and Robinson [1925] compared the results of p_H determinations on dog, sheep, and human serum or plasma, determined gasometrically and electrometrically at 38°, with colorimetric results obtained at 38° and

at 20°. They concluded that the colorimetric reading of a serum or plasma, obtained by the technique of Hastings and Sendroy, could not be relied upon.

Myers and Muntwyler [1928], using the micro-colorimetric method of Myers, Schmitz and Booher [1923], state that "colorimetric and electrometric determinations of the p_H made on the same sample of blood agree within 0·02 p_H when both determinations are made at the same temperature." Furthermore, there seemed to be no apparent reason for the observed variations in the C correction. The results of 51 protein determinations which they carried out indicated that a change in protein concentration is not a factor. This is in agreement with Marrack and Smith [1924], who found that the Cullen correction did not change with abnormalities in plasma proteins.

Errera, Reding and Slosse [1929] have compared the results of plasma p_H determinations at 38°, obtained by the colorimetric method of Hastings and Sendroy, with those obtained by the use of the glass electrode. The results of 30 determinations on the plasma of normal and cancerous patients indicate that the colorimetric method is reliable.

These findings are further supported by those of Bayliss, Kerridge and Verney [1926], who found excellent agreement between results obtained by the colorimetric, hydrogen electrode and glass electrode methods.

The present investigation.

To test the reliability of the colorimetric method, it was decided to compare colorimetric readings made at 38° on plasma with p_H values obtained by the use of the glass electrode on the same material and for this purpose the apparatus described elsewhere by Fosbinder [1930] and by Fosbinder and Schoonover [1930] was used.

If plasma is diluted 20-fold, thereby reducing the partial pressure of the free CO_2 to approximately 1/20 of its original value, it is possible to carry out electrometric measurements on this material at 38° without loss of CO_2 by covering the surface of the liquid with paraffin oil. Errera, Reding and Slosse [1929] apparently found this procedure to be unsatisfactory. However, Hastings and Sendroy [1924] observed no appreciable shift in the p_H of bicarbonate buffers of physiological strength when they were diluted 5- to 20-fold with 0·154 *M* sodium chloride.

Procedure and results.

The p_H of plasma at 38° was determined by the colorimetric method of Hastings and Sendroy [1924]. The vacuum tube potentiometer developed by one of us was used to determine glass electrode potentials. Glass electrodes, of the form described by MacInnes and Dole [1929, 1930], and identical in composition, were employed.

The electrometric p_H values were determined with the use of the following cell

Hg | Hg_2Cl_2 | saturated KCl || buffer or plasma | glass | 0·1 *N* HCl, AgCl | Ag.

In order to diminish the liquid junction potential between the saturated potassium chloride and the diluted plasma, 0·15 *M* KCl containing phenol red was employed as the diluent. Adjustment of the potassium chloride indicator solution, which served as the plasma diluent in both the colorimetric and glass electrode methods, was accomplished by adding to it a solution of 0·01 *N* NaOH, containing in addition potassium chloride and phenol red in the same concentrations as those of the indicator solution. This procedure avoided the possibility of any error due to dilution. The maximum buffer value of the indicator solution was 0·10 *mM* of NaOH per unit p_H per litre, which is approximately 1/50 of the buffer value of the plasma. Fortunately, it was possible to substitute potassium chloride for sodium chloride, for Hastings and Sendroy [1929] found that the dissociation constants of phenol red in the two solutions are identical at 20°, therefore, we may use the sodium chloride value at 38°, 7·65, for potassium chloride.

The bicolour standards were compared with a recently prepared set of standards of known log *R* values. The results of this comparison are given in Table I.

Table I. *Log* R *and* p_H *values of phenol red bicolour standards.*

Apparent p_H values of indicator pairs	log *R*	Corrected p_H values of indicator pairs
7·15	−0·49	7·16
7·20	−0·45	7·20
7·25	−0·40	7·25
7·30	−0·35	7·30
7·35	−0·30	7·35
7·40	−0·25	7·40
7·45	−0·20	7·45
7·50	−0·15	7·50
7·55	−0·09	7·56
7·60	−0·05	7·60
7·65	0·00	7·65
7·70	+0·08	7·73

The procedure employed in determining the glass electrode p_H values was a comparison of the potentials observed with the diluted plasma with the potential of a *M*/15 phosphate buffer solution of known p_H. The p_H value of the buffer solution was determined daily both colorimetrically and electrometrically. The electrometric standard was 0·1 *N* HCl, which was assumed to have a p_H value of 1·08 at 38°. To calculate the p_H of the plasma the following equation was used

$$p_H \text{ (plasma)} = p_H \text{ (buffer)} + \frac{E-e}{0{\cdot}0617},$$

where E = the potential observed with diluted plasma, and e = the potential observed with the buffer.

The technique employed in the determination of the p_H of the diluted plasma was as follows. 2 cc. of 0·15 *N* KCl indicator solution, adjusted to a p_H of approximately 7·4, were added to the electrode cell, and the glass electrode immersed in the solution. A layer of paraffin oil, approximately

7 mm. in depth, was then placed over the surface of the liquid, sealing the solution from the air. One-tenth of a cc. of plasma was then added to the diluent beneath the surface of the oil and the solution thoroughly stirred with a small glass rod. The plasma was introduced from a tuberculin syringe, so much plasma being drawn into the syringe that the colorimetric and the electrometric readings could be made on the same sample. There was no evidence of CO_2 escaping from the diluted plasma, for the potential remained constant over a considerable period of time.

The results of 28 unselected determinations of normal and pathological plasmas are given in Table II.

Table II. p_H *values of diluted plasma obtained by the colorimetric and glass electrode methods.*

Description of plasma	$p_{H38°e}$	$[p_H]_{38°}$	$[p_H]_{38°} - p_{H38°e}$	$([p_H]_{38°} - c_{p_{K'}}) - p_{H38°e}$
Normal	7·38	7·39	+0·01	0·00
	7·38	7·39	+0·01	0·00
	7·41	7·42	+0·01	0·00
	7·40	7·42	+0·02	+0·01
	7·34	7·35	+0·01	0·00
	7·37	7·37	0·00	−0·01
	7·39	7·39	0·00	−0·01
	7·41	7·39	−0·02	−0·03
	7·38	7·40	+0·02	+0·01
Normal plasma allowed to stand 24 hours	7·35	7·36	+0·01	0·00
	7·28	7·29	+0·01	0·00
	7·29	7·29	0·00	−0·01
	7·20	7·19	−0·01	−0·02
	7·28	7·29	+0·01	0·00
Cancerous	7·37	7·37	0·00	−0·01
	7·31	7·32	+0·01	0·00
	7·39	7·38	−0·01	−0·02
	7·38	7·37	−0·01	−0·02
	7·39	7·39	0·00	−0·01
	7·39	7·38	−0·01	−0·02
	7·39	7·39	0·00	−0·01
	7·41	7·42	+0·01	0·00
	7·42	7·42	0·00	−0·01
	7·35	7·36	+0·01	0·00
	7·40	7·38	−0·02	−0·03
	7·40	7·38	−0·02	−0·03
Cancerous?	7·36	7·35	−0·01	−0·02
Goitre	7·36	7·39	+0·03	+0·02
Average deviation	—	—	+0·002	−0·008
Maximum deviation	—	—	0·03	0·03

In column 5 is given the deviation between the two methods when the dissociation constant of phenol red is corrected to $\log R = 0$. [See Hastings and Sendroy 1929, p. 238.]

Discussion.

The remarkably close agreement between the results of the two methods

$$[p_H]_{38°} - p_{H38°e} = + 0{\cdot}002 \text{ or } [p_H]_{38°} \text{ (corr.)} - p_{H38°e} = - 0{\cdot}008$$

shows that there are no deviations which cannot be accounted for otherwise than by experimental error. Our observations on diluted plasma are in agreement with the results obtained on whole plasma by Hastings and Sendroy [1924], Myers and Muntwyler [1928], Errera, Reding and Slosse [1929], and others.

Most authors are in agreement that the normal p_H of the blood lies between 7·30 and 7·40. However, in the case of normal plasma, one would not expect to find so great a range of deviation. The average of 9 electrometric determinations made by us on the p_H of plasma of normal individuals is 7·38, with a deviation from the mean of + 0·005 p_H. Cullen and Robinson [1923], in 27 determinations of the p_H of normal plasma, found a variation from 7·28 to 7·41. Nevertheless, it is significant that 21 of the 27 determinations lay between 7·35 and 7·40. Quoting Reding [1928], the average normal value obtained by the electrometric method was found by Michaelis to be 7·35; Cullen, 7·35; Van Slyke, 7·35; Vincent, 7·34. Normal values obtained by the colorimetric method are: Cullen and Hastings, 7·36; Bigwood, 7·36; Cullen and Robinson, 7·35. Corran and Lewis [1924], using the quinhydrone electrode, obtained an average value of 7·33 on the whole blood of nine normal patients at 37°. If we add to this value 0·06 p_H, which is the protein error of the electrode, we find that the average value for normal blood is 7·39. Reding [1928], in the study of 25 normals, arrives at an average value of 7·36 with the use of the colorimetric method. Results obtained by other workers in our laboratory[1] on 15 normals yield a value of 7·37. From these studies it appears very probable that the average normal p_H of the blood lies between 7·35 and 7·38.

No attempt is made here to compare the p_H of the blood of cancer patients with that of the blood of normal subjects, since all of the samples of blood which we received were drawn from patients who had already received a considerable amount of treatment.

Our results are not influenced by the fluctuations of the acid-base balance during the day, as observed by Cullen and Earle [1929], since all our determinations were made upon blood which was drawn at approximately the same hour each day.

Summary.

1. The colorimetric method of Hastings and Sendroy may be used with accuracy for the determination of the p_H of blood or plasma at 38°.

2. Determinations carried out at 38° on 28 samples of diluted plasma from

[1] The authors wish to thank Miss Gladys Woodward and Miss Edith Fry for making these figures available to them.

cancerous and normal subjects reveal no discrepancy between the colorimetric and glass electrode methods.

3. The p_H of normal plasma or blood lies between 7·35 and 7·38.

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CCIV. HEART BLOCK IN PIGEONS, CURATIVE FACTOR.

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A condition of sinus bradycardia and heart block, which develops in pigeons fed on a diet of polished rice, was recently described by Carter and Drury [1929]. It was shown that the cardiac irregularity manifested itself at an earlier period after the commencement of rice feeding than head retraction, and, apart from loss of weight, was in fact one of the earliest indications of dietary deficiency in the pigeon. It was also observed that the irregularity was completely abolished by vagal section, or by atropine, and the suggestion was made that it was due to an over action of the vagus nerve.

The present work was undertaken with a view to elucidating the factor or factors, the absence of which from the diet of polished rice gives rise to heart block.

Experimental.

In order to have a standard for comparison, the heart rate has been recorded in a number of normal pigeons, using the stethoscope for this purpose. This method has been found to be satisfactory when compared with records obtained with the electrocardiograph. .

Buchanan [1909] found the heart rate in 3 normal pigeons to be 141, 186, and 225 per minute respectively.

My own observations have confirmed the variability in the heart rate not only as between different individuals, but in the same bird examined under the same conditions from day to day. This is true even when disturbing factors such as excitement or slight muscular movement are excluded. Table I shows the heart rate of 7 normal birds, chosen at random, examined on different days.

Table I.

	Heart rate per minute					
Bird	1	2	3	4	5	6 days
1	232	232	264	200	182	—
2	260	288	240	288	280	—
3	240	264	212	216	212	212
4	224	216	224	220	198	212
5	216	188	208	212	208	170
6	160	166	158	192	188	188
7	212	184	182	186	192	—

In the experiments to be described only birds whose normal heart rates exceeded 170 per minute have been employed. When placed on a diet of polished rice, there is a progressive slowing of the heart rate (80–130 beats per minute), and in the majority of cases irregularity of rhythm is present at some stage. In some of these the irregularity is succeeded by a slow regular rhythm in which there is complete dissociation between auricle and ventricle, as can be demonstrated by the electrocardiograph. During exercise the irregularity disappears but during an ensuing period of rest it reappears, to be followed in some cases by a period of slow regular rhythm.

Exp. 1. It was thought that deficiencies in the protein or inorganic salt content of polished rice might be responsible for the development of heart block, and with a view to eliminating these possibilities a group of 4 birds was placed on a synthetic diet having the following composition:

Glaxo "casein" (vitamin B-free)	15 %
Rice starch	75
Fresh butter	5
McCollum's salt mixture	5

Regular daily doses of cod-liver oil were given in addition in order to ensure an adequate supply of fat-soluble vitamins. The powdered diet was mixed with water to give a granular consistency and was given freshly prepared each day. The birds readily ate the food supplied although in the later stages, as in the case of rice feeding, the appetite rapidly deteriorated. By the end of the 21st day all the birds had developed a well marked heart block, and death occurred in each case within 35 days. No head retraction was observed, but in one bird wing drop developed on the 21st day. Table II shows the data for one bird typical of the group.

Table II.

No. of days on diet	Weight g.	Heart rate	Remarks
0	412	240	Regular rhythm
5	387	216	,,
10	363	180	Dropped beats
22	305	132	Irregular
25	290	136	,,
31	267	132	,,
33	—	—	Dead

It is evident from this experiment that any deficiency of protein, fat, or inorganic salts in a basal diet of polished rice is not related to the onset of heart block, since a diet complete in these respects does not prevent or delay its appearance. Moreover, the condition appears in its typical form even when an adequate supply of the fat-soluble vitamins is consumed.

A further series of experiments was undertaken with a view to ascertaining whether lack of one or more of the factors of the vitamin B complex played any part in the development of heart block. Both curative and protective tests were employed for this purpose. In some experiments birds were fed on

a polished rice diet until heart block was well established. The diet was then supplemented with daily doses of a fraction containing one or more vitamin factors, given by mouth or by injection. Observations of body weight and of the rhythm and frequency of the heart were recorded daily. In some experiments the protective test was employed, the birds being fed on a polished rice diet which was supplemented from the beginning of the experiment with test doses. The source of vitamin B_1 employed was the concentrate prepared according to the method of Kinnersley and Peters [1927]. Vitamin B_2 was given in the form of marmite heated at p_H 9·0 at 115° for 1 hour. In some experiments untreated marmite was used. This has been shown by Reader [1930] to contain a factor B_4, which is not identical with the Williams-Waterman factor B_3, in addition to vitamins B_1 and B_2.

Effect of vitamin B_1.

Exp. 2. A group of 7 birds was placed on a diet of polished rice, and each bird was given by mouth daily 0·1 cc. of the standard concentrate equivalent to 4 pigeon day doses of the antineuritic vitamin. The acid alcohol extract was heated for 2 minutes on a water-bath to remove the alcohol, carefully neutralised and diluted to 5 cc. before administration. The birds survived for varying periods up to 80 days and throughout the period of survival showed a gradual loss of weight. Within 13 days of the beginning of the experiment every bird showed typical heart block. Table III shows the data for this period of the experiment. In a number of cases prior to death the slow, irregular rhythm of the heart became very rapid and regular, indicating the sudden and final failure. The daily dose of the vitamin was at least twice the amount necessary to protect the pigeon against polyneuritis, but was without effect in protecting the bird against heart block.

Table III.

	Pigeon 64		Pigeon 58		Pigeon 69		Pigeon 66		Pigeon 110		Pigeon 115		Pigeon 122	
Day	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate
1	382	204	390	224	440	220	432	204	390	200	452	174	380	208
7	350	144	360	124	410	160	400	170	370	162	430	170	364	156
13	326	98	342	110	364	132	384	100	332	90	396	128	312	128

The progressive fall of heart rate has become very marked by the 13th day. On the 7th day irregularity of rhythm was noted in 2 birds. By the end of the 13th day 5 birds exhibited marked irregularity.

In several birds which had been allowed to develop heart block the injection, intravenously or intramuscularly, of relatively enormous doses of antineuritic vitamin entirely failed to effect a cure. Table IV shows the absence of curative effect after the injection of 0·6 cc. of an antineuritic concentrate equivalent to 18 pigeon day doses repeated on three successive days.

In two birds which had developed heart block a cannula was tied into the jugular vein under urethane anaesthesia, and doses of vitamin B_1, equivalent to 2 pigeon day doses and 6 pigeon day doses respectively, were injected, the

extract being washed into the vein with a small quantity of saline solution. Observations at short intervals over a period of 4 hours failed to reveal any improvement of the heart condition.

Table IV.

Date		Heart rate	Rhythm	Remarks
April 19.	11.12 a.m.	124	Regular	Bradycardia
	6.5 p.m.	104	,,	
,, 20.	12.8 ,,	120	,,	
	12.15 ,,			Intramuscular injection 0·6 cc. vitamin B_1
	3.5 ,,	116	Dropped beats	Heart block
	6.26 ,,	110	Regular	
,, 21.	11.56 a.m.	116	,,	
	3.10 p.m.			Intramuscular injection 0·6 cc. vitamin B_1
	6.50 ,,	128	,,	
,, 22.	12.0 noon	128	,,	
	3.0 p.m.			Intramuscular injection 0·6 cc. vitamin B_1
	6.30 ,,	136	,,	
,, 23.	11.53 a.m.	134	,,	
	3.52 p.m.	124	Irregular	Heart block. No recovery

Effect of vitamins B_1 and B_2.

Exp. 4. A group of 11 birds was placed on a diet of polished rice which was supplemented by daily doses of vitamin B_2. The vitamin was supplied in the form of marmite suspended in water. This had been previously heated to a temperature of 115° for 1 hour at p_H 9·0, and was neutralised before administration. This treatment results in complete destruction of vitamin B_1 without serious destruction of vitamin B_2 [Reader, 1930].

Seven birds received a daily dose of 0·5 g. of the autoclaved marmite. Each bird received in addition a daily dose of cod-liver oil. The remaining 4 birds received daily 1·0 g. of the supplement, and cod-liver oil. By the end of the 13th day heart block was established in every bird.

Exp. 5. A group of 8 birds on a polished rice diet received daily 1·0 g. of marmite autoclaved as described above, together with 0·1 cc. standard vitamin B_1 concentrate, equivalent to 4 pigeon day doses of the antineuritic factor. Cod-liver oil was given in addition. Seven birds developed heart block at varying periods up to the 18th day; the remaining bird showed no cardiac abnormality up to the time of death, which occurred on the 49th day.

It is evident that vitamin B_2 is incapable of protecting the pigeon against the development of heart block when the bird is fed on a basal diet of polished rice, even when supplemented by the antineuritic factor. It may be noted that fall of body weight occurred under these conditions.

Effect of marmite.

Several experiments were performed in which untreated marmite was employed as a supplement to a polished rice diet. In one of these marmite was given by mouth in large doses (2·0–4·0 g. daily) over a period of a few days. In another experiment a smaller dose (1·5 g. daily) was given over a

period of many weeks. With daily doses exceeding 2·0 g. difficulty was experienced owing to the fact that in some cases part of the marmite was vomited, and in most cases diarrhoea was set up owing to its high salt content.

Exp. 6. A bird which had developed heart block on a diet of polished rice was given in addition 2·0 g. untreated marmite daily in 5·0 cc. water for 5 days. This dose of marmite is usually found to produce in an avitaminous pigeon an initial daily increase in weight of about 10 g. which diminishes steadily until after about 5 days the weight is again stationary. In the present case the heart condition failed to show any improvement. This is seen in Table V.

Table V.

Date	Heart rate	Rhythm	Remarks
April 18. 11.45 a.m.	176	Irregular	Heart block
5.55 p.m.	132	„	
„ 19. 11.27 a.m.	128	Regular	
6.8 p.m.	120	„	2 g. marmite by mouth
„ 20. 12.15 „	160	„	
6.42 „	140	„	2 g. marmite by mouth
„ 21. 11.22 a.m.	216	Irregular	
6.32 p.m.	160	„	2 g. marmite by mouth
„ 22. 12.40 „	176	„	
7.2 „	144	Regular	2 g. marmite by mouth
„ 23. 12.40 „	160	„	
„ 24. 12.40 „	156	Irregular	Heart block. No recovery, but some improvement in heart rate

Exp. 7. Six birds, after a preliminary period on polished rice, were given daily in addition 1·5 g. marmite by mouth, and 0·5 cc. cod-liver oil once weekly. This diet was continued for a period of 91 days, records of the heart condition and body weight being made at intervals of 2 or 3 days. Table VI summarises the data of this experiment in the case of 4 birds.

Table VI.

	Pigeon 3		Pigeon 47		Pigeon 76		Pigeon 152	
Day	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate	Weight g.	Heart rate
0	250	104	318	120	322	92	228	100
17	268	108	346	148	322	120	264	188
30	302	188	346	184	347	168	272	164
64	300	142	352	196	364	180	292	232
85	318	152	380	232	379	184	316	304
	Marmite discontinued. Whole wheat diet							
116	411	166	468	272	484	240	—	—

It will be seen that throughout the whole period of marmite feeding the recovery of body weight was gradual and incomplete, though it was noticeable that the general condition of the birds showed a very great improvement. Of the cases detailed in Table VI recovery of normal rhythm and frequency of the heart occurred in three birds within 60 days. On the other hand,

Pigeon 3 showed occasional periods of heart block up to the 85th day, and even on a whole wheat diet the heart did not recover its normal rate. The remaining two birds of this group died on the 22nd and 30th day respectively after commencing marmite feeding without showing any recovery from heart block. It is evident that marmite in doses of 1·5 g. has very little curative action on heart block even when administered for long periods.

Exp. 8. Three birds which had been fed for 2 weeks on whole wheat were placed on polished rice, supplemented by 1·5 g. marmite daily. This diet was continued for 36 days. The heart in all the birds remained normal both as regards rhythm and frequency throughout this period. Marmite appears to be effective in protecting the pigeon against the development of heart block though relatively inefficient as a curative agent.

Curative action of yeast.

Baker's yeast proved to be much more effective in the cure of heart block than marmite.

Exp. 9. Two birds were fed on polished rice for 18 days. Subsequently they received in addition daily doses of baker's yeast in amounts of 2 g. (dry weight 0·6 g.), and 6 g. (dry weight 2·0 g.) respectively. With the smaller supplement there was a definite response of the heart. The rate increased and phases of irregularity of rhythm were less frequent. The beneficial effect was, however, temporary and had more or less disappeared within 24 hours of giving the yeast. With the larger dose the curative action was more striking (Table VII).

Table VII.

Date	Heart rate	Weight g.	Remarks
Pigeon 1:			
May 1. 10.50 a.m.	104	—	Slow heart rate. Rhythm regular
„ 2. 12.17 p.m.	108	256	
12.30 „	—	—	2 g. yeast by mouth
3.0 „	108	—	No change in heart
„ 3. 12.15 „	232	—	Recovery
12.20 „	—	—	2 g. yeast by mouth
„ 4. 12.0 noon	128	262	Slow rate. Dropped beats.
12.5 p.m.	—	—	2 g. yeast by mouth
„ 5. 10.38 a.m.	180	—	Recovery
„ 6. 10.45 „	152	268	
3.15 p.m.	116	—	Relapse to slow rate with occasional irregularity
Pigeon 2:			
May 1. 11.15 a.m.	104	258	Slow heart rate. Rhythm regular
11.30 „	—	—	6 g. yeast by mouth
„ 2. 11.15 „	400	262	Recovery, heart rate very rapid
„ 4. 11.0 „	136	264	Relapse to slow rate
„ 4. 11.10 „	—	—	6 g. yeast by mouth
„ 5. 10.30 „	312	271	Recovery

Brewer's yeast was found to be as effective as baker's yeast in restoring normal function of the heart.

Exp. 10. A group of 6 pigeons, after a depletion period on polished rice varying from 11 to 25 days, were given subsequently daily doses of brewer's yeast of 6·0 g. (dry weight 2·1 g.). There was a rapid improvement in the heart, and normal function was restored in 1–6 days. In two of these the recovery took place within 24 hours. Table VIII gives the data for these birds.

Table VIII.

Days on rice		Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6
0	Heart rate	180	196	192	208	216	188
	Weight (g.)	432	445	410	411	394	448
5	Heart rate	160	184	156	160	176	196
	Weight (g.)	390	411	370	328	332	381
11	Heart rate	140*	144*	120*	—	—	184
	Weight (g.)	323	361	312	—	—	336
13	Heart rate	134	140	136	144	176	—
	Weight (g.)	350	370	345	291	268	—
14	Heart rate	184	144	152	136*	—	—
	Weight (g.)	341	365	342	284	—	—
15	Heart rate	—	164	170	190	—	—
	Weight (g.)	—	368	345	305	—	—
16	Heart rate	208	170	190	192	140	—
	Weight (g.)	342	370	342	287	253	—
17	Heart rate	—	—	—	—	124*	—
	Weight (g.)	—	—	—	—	248	—
18	Heart rate	200	—	196	176	174	—
	Weight (g.)	354	—	353	320	270	—
24	Heart rate	—	—	—	200	—	96
	Weight (g.)	—	—	—	363	—	275
25	Heart rate	198	188	196	—	232	80*
	Weight (g.)	379	392	375	—	297	267
26	Heart rate	—	—	—	—	—	152
	Weight (g.)	—	—	—	—	—	280
32	Heart rate	216	196	198	192	232	160
	Weight (g.)	371	391	381	372	321	327

* Doses: 6·0 g. brewer's yeast given daily thereafter.

Curative action of wheat.

Exp. 11. Following a preliminary period of 18 days on polished rice a group of pigeons was fed daily supplements of 2, 4, 10 and 20 g. of whole wheat. All birds exhibited definite heart block before administration of wheat. The birds receiving rations up to 10 g. daily of wheat showed no improvement of

Table IX.

	Pigeon 1			Pigeon 2	
Date	Heart rate	Remarks	Date	Heart rate	Remarks
April 18	128	Irregular rhythm	April 21	112	Heart block
„ 19	128	12 g. wheat daily	„ 21	—	20 g. wheat daily
„ 20	148		„ 22	152	No recovery
„ 21	144		„ 23	192	Recovery
„ 22	132		„ 24	188	
„ 23	160		„ 25	208	
„ 24	140	Irregular rhythm			
„ 27	170				
„ 28	160	Definite improvement			

the cardiac condition over a period of 6 days. A supplement of 12 g. daily of wheat produced some improvement by the end of 8 days. Birds fed daily doses of 20 g., constituting about 60 % of the daily food intake, showed complete recovery of the heart in about 3 days. Table IX shows the data for two of these birds.

Exp. 12. A group of 4 birds was placed on a diet of wheat which had been previously heated at 100° for 5 hours in a steamer. This treatment involved some destruction of the antineuritic factor, since three of the birds developed head retraction on the 60th, 65th and 76th day respectively. The birds received cod-liver oil at regular intervals. During the first 15 days there was only slight loss of weight, but later this loss became rapid. By the end of the 22nd day all birds had developed heart block. Table X shows the data for one of these birds.

The substance responsible for the curative effect on the heart which is present in wheat is therefore destroyed by this treatment.

Table X.

Days on steamed wheat	Weight g.	Heart rate	Remarks
0	520	240	Normal heart action
13	486	156	Irregular rhythm. Heart block
16	486	140	
22	480	88	
46	315	160	No recovery

Exp. 13. Powdered wheat was thoroughly extracted with ether five times. Three birds were placed on polished rice until heart block had become well established at the end of the 18th day. Two of the birds were then fed daily on the extracted wheat, which was moistened with water to give a granular consistency. There was an immediate rapid increase in weight together with a great improvement in the general condition of the birds. The heart became normal in rhythm and frequency within 3 days. The third bird was given daily doses of the dark yellow oil obtained on extraction of the original wheat, containing the fat-soluble vitamins A, D, and E. The bird continued to lose weight and developed head retraction on the 23rd day. No improvement occurred in the condition of the heart. It is evident that extraction with ether does not remove the curative substance from wheat (Table XI).

Table XI.

	Pigeon 1			Pigeon 2			Pigeon 3		
Date	Weight g.	Heart rate	Remarks	Weight g.	Heart rate	Remarks	Date	Weight g.	Heart rate	Remarks
Nov. 22	308	92	Heart block	272	92	Heart block	Nov. 20	300	80	Heart block
„ 22	—	—	Ether-extracted wheat	—	—	Ether-extracted wheat	„ 20	—	—	1 cc. ethereal wheat extract daily
„ 25	351	172	Recovery	306	192	Recovery	„ 22	294	108	
„ 30	432	196		388	272		„ 25	—	148	
							„ 27	260	120	Head retraction. No improvement in heart action. Died

Discussion.

The experiments described in this paper indicate that whole wheat and yeast contain a factor, or factors, essential for the normal metabolism and nutrition of the pigeon, the absence of which from polished rice leads to the development of heart block, among other disorders.

The evidence here presented would appear to indicate that the well-known deficiencies of the basal constituents of polished rice are not related to the development of heart block. A diet complete in respect of its content of protein, fat, carbohydrate and inorganic salts, even when supplemented by cod-liver oil, fails to protect the pigeon against this disorder.

It is clear also that the disorder of metabolism which manifests itself in heart block is not identical with that underlying the onset of polyneuritis. The administration of vitamin B_1 in a dose more than sufficient to clear up signs of polyneuritis, even when this is repeated daily for several weeks, is without effect on the heart. This is true also when vitamin B_2 is given in addition. The fat-soluble vitamins A, D and E, present in the ether-soluble material extracted from whole wheat, and in cod-liver oil and butter, are incapable of clearing up an established heart block, or of preventing its development.

Williams and Waterman [1928] described a factor present in wheat and in yeast which is essential for the normal nutrition of the pigeon but not for the rat. Until it has been found possible to obtain this factor free from the other constituents of the vitamin B complex it will be impossible to decide whether this is identical with the substance present in wheat and yeast on which the normal function of the heart depends. The similarity in the distribution of these substances and their thermolability may, however, be noted. Drury and Harris have recently reported a condition of sinus bradycardia without bundle block in rats fed on a vitamin B-free diet. In the extensive literature on the cardiac manifestations of human beri-beri there is no evidence of heart block even when electrocardiographic records have been taken of the heart [Aalsmeer and Wenkebach, 1929; Keefer, 1930].

In conclusion I wish to express my sincere thanks to Prof. Peters for his continued interest and advice and for the opportunity afforded me of making use of his antineuritic preparations. I am also indebted to Dr A. N. Drury for certain electrocardiographic records and for his assistance with some of the operations.

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CCV. A QUANTITATIVE COMPARISON OF THE CURATIVE ACTIVITY OF TORULIN (VITAMIN B_1) UPON THE ADULT PIGEON AND THE ADULT WHITE RAT.

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The standard method of testing for vitamin B_1 in this laboratory has been a curative and protective test upon pigeons, as laid down by Kinnersley, Peters and Reader [1928]. It is known from the work of Chick and Roscoe [1927] that torulin preparations made by our methods are highly curative to rats suffering from polyneuritis, and that they can be used as a growth factor by the growing rat. This has been confirmed by Reader [1930] as an incident in the elaboration of a technique for testing for Reader's vitamin B_4. In spite of the growth-promoting effect of these preparations, there is as yet no strict proof that the vitamin B_1 under consideration in our concentrates is the same factor for the pigeon and the rat. We felt that if it could be shown that the curative and protective effect for the rat in several different preparations ran parallel to that for pigeons, it would constitute a proof that the factor concerned was essentially the same for both animals. Accordingly we have compared the vitamin B_1 (torulin) preparations from this point of view. A preliminary account of the experiments appeared in the Harben lectures [Peters, 1929, 1930], stating that approximately the same dose was required to cure and protect the rat as in the case of the pigeon. About the time of publication of these lectures, a paper by Smith [1930] appeared, in which a curative test is employed. The author uses as a standard the minimum dose of a preparation required to cure a rat suffering from polyneuritis. In an accompanying paper, Reader [1930] deals with the question of the symptoms in these tests. A rough comparison of Smith's tables with our results suggests that our method with rats gives rather more regular answers, but comparative tests would have to be made to determine the relative merits of the two methods.

Experimental.

Pigeon tests. These have been made by the method previously described. The objections put forward by Smith to the pigeon-cure test have been dealt with in our previous communication [1928]. The first objection that spon-

taneous cures occur, we have dealt with in full. His second objection is as follows: "Cures effected in polyneuritic pigeons by the administration of the antineuritic substance have often been noted to be little or not at all sustained, and instances have been known in which birds were totally refractory to treatment during subsequent attacks." We are not quite certain that we understand what is here considered. Out of a very large experience of these birds (amounting to some 1000 birds a year for the last 6 years), we have come across few instances (certainly nor more than 1 %) in which a genuine vitamin B_1 extract would not cure a bird suffering from apparently genuine symptoms. Such cases are evidently not suffering from vitamin B_1 deficiency, and that is why they do not react to the tests. It is unfortunate that there is at present no means of easily distinguishing such cases. Many other physiological disturbances will induce head retraction in pigeons, as for example an overdose of insulin. Such instances have been collected elsewhere [Peters, 1929, 1930]. It conveys a false idea of the facts to think that enough of these cases appear under controlled feeding conditions to disturb the tests seriously. Further, it must be recognised that it is unlikely even that the rat will not be occasionally abnormal. Polyneuritic symptoms in the rat resemble rather closely those of an overdose of insulin in the mammal. The important point in using a complex symptom in animals as a means of testing is to eliminate as far as possible similar disturbances due to other causes. This we know to be a truism, but it is often overlooked. Smith's third objection as to the temporary cures produced by other substances is not a serious one in our opinion. It is in the main eliminated by the preliminary dosing, and if necessary it can be eliminated completely by the method recently described by Gulland and Peters [1929]. In our experience the only one of these reagents mentioned which we have ever found to cure is histamine. The fact that any test dose of histamine which we have given of late has not induced cure has led us to suppose that the histamine cure is due to occasional traces of impurities in commercial samples.

The difficulties to be found in applying the pigeon curative and preventive test are those inherent in all biological tests, namely the innate biological variation of the animals. In the case of the pigeon, the absence of a standard strain probably makes this wider than for an inbred strain of rat. To give some concrete idea of this, in Table I, out of the 20 tests upon a given dose of the average value of 4·4 days, 10 % of the bird tests gave values under half the average, and 10 % values approximately twice the average. With preparation B, a similar kind of result was obtained. We do not think that the values shown by Smith in his Table I give any better agreement than this, and doubt whether for many preparations the tests can be made with a less variation. We would record it as our impression however that we have had in our hands preparations which have given much more consistent results than these; at present we do not pretend to understand this. C, for instance (Table I), has been tested indirectly many times, and has seldom given less

than 3 or more than 5 days. The results obtained for the rats, by a precisely similar method of testing, form valuable independent evidence of the validity of our pigeon methods.

Rat tests. Adult albino rats (bred in this department) of approximately maximum weight (males 250 g., females 170 g.) were fed on a diet of caseinogen (20 parts), rice starch (70 parts), agar-agar (2 parts), salt mixture (5 parts), cod-liver oil (3 parts) and alkaline-autoclaved yeast extract (6 parts). This last item was supplied as a source of vitamin B_2 [*cf.* Reader, 1930]. In the course of 21–24 days the rats were found to decrease in weight to about 100–120 g. Convulsions then occurred rather suddenly accompanied by partial paralysis, especially of the hind legs. The feet are always splayed out, and often the head is held on one side. The animal may live for a few hours in this condition, but frequently death occurs in about 30 mins. It is advisable to give the test dose of torulin (vitamin B_1) as soon after the onset of symptoms as possible, as the throat often becomes paralysed, so making dosing by mouth impossible. The test doses were always given by mouth from a pipette. A marked improvement may often be noticed within 20 minutes of the time of dosing the animal and always in 2–3 hours, especially if not less than a three-day dose is given. The second onset of convulsions occurs on the 2nd, 3rd or *n*th day according to the size of dose given. Convulsions and paralysis of the hind limbs appear to be the true polyneuritic symptoms and are cured by vitamin B_1 in its purest form. The symptoms due to lack of vitamin B_1 are usually accompanied by those due to deficiency of vitamin B_4, such as swollen paws, ataxia, etc. The latter are not removed by dosing with vitamin B_1 and can be used for the assay of vitamin B_4 [see Reader, 1930].

The range of variation of the rat curative test for vitamin B_1 may be judged from the figures for a given preparation (preparation B in Table I) where 12 rats were used; the maximum value obtained was 6, minimum 4, and the average (for 12) 4·5.

Results. We have collected in Table I the results of tests upon pigeons and rats of different preparations, varying in activity from one with a pigeon-day dose of 0·025 mg. to one of 1·2 mg.

Table I.

	Dose of vitamin B_1 prep. cc.	No. of day doses		Activity mg.
		Pigeons	Rats	
(A)	1·0	4·4 (20)	4 (3)	1·2
(B)	0·1	2·9 (37)	4·5 (12)	0·13
	0·15	—	6 (2)	0·13
(C)	0·10	3·8 (8)	3·5 (5)	0·10
(D)	0·08	5·0 (3)	4 (6)	0·25

The activity is expressed in mg. organic solids per daily pigeon dose.

In the above table it will be seen that the agreement is reasonably close except in the case of preparation B. In this the preparation appears to be

1·5 times more active for the rat than for the pigeon. When it is remembered that there is no reason why there should be any correspondence between these figures apart from identity of the factors concerned and that biological tests are subject to rather wide variations unless very large groups of animals are taken, we think that the figures leave no doubt in the mind that we are dealing with the same factor in the rat and the pigeon.

SUMMARY.

Parallel tests upon pigeons and rats by a curative and protective method with torulin (vitamin B_1) preparations of different degrees of purity indicate that the B_1 factor for the two animals is identical, and show that the dose of vitamin B_1 required by each animal is approximately the same.

Thanks are due to the Medical Research Council for a personal grant to two of us (V. R. and H. W. K.) and for a grant for expenses. We are grateful to W. Wakelin and M. Kempson for assistance with the tests.

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CCVI. THE CURATIVE ACTIVITY OF THE ANTINEURITIC VITAMIN OF RICE.

By BARD COENRAAD PETRUS JANSEN, HENRY WULFF KINNERSLEY, RUDOLPH ALBERT PETERS and VERA READER.

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In 1926 Jansen and Donath [1926] published a method of isolating a crystalline vitamin from rice polishings capable of protecting bondols from polyneuritis in doses of 0·002 mg./day, and pigeons from loss of weight upon a diet of polished rice together with meat powder in doses of 0·01 mg./day. Since then it has seemed desirable to determine the activity of the crystals in terms of other methods of testing for vitamin B_1.

This paper records tests made in the Department of Biochemistry, Oxford, upon material prepared in Amsterdam.

Experimental.

Rice vitamin. The crystallised specimen of vitamin hydrochloride used for these experiments was prepared from rice polishings and had a melting point of 250°. For the purpose of the experiment it was dissolved in $N/1000$ HCl, to which 15 % ethyl alcohol was added, and kept in cold store while the tests were being carried out.

Tests. These were made by the curative method. The curative pigeon tests were carried out by the technique described by Kinnersley, Peters and Reader [1928] and the curative rat tests by a method to be shortly described by Kinnersley, Peters and Reader. The growth-promoting effects upon *Streptothrix corallinus* were examined by the original technique described by Orr-Ewing and Reader [1928].

It will be seen that the three methods of testing are not absolutely consistent, though they agree that the activity of the crystals lies between 0·005 and 0·009 mg. This is an activity higher than that yet obtained for torulin concentrates. It is to be noted that there is a rather considerable variation in the pigeon tests, all of which have been here included. The variation is larger than that to which we are accustomed with yeast preparations. We are unable at present to account for this; the tables indicate that it is not related

to the time of year; but the average value of 0·009 mg. by mouth and 0·007 mg. by injection is definitely more active than any yeast preparation which we have so far tested. It is also close to the value of 0·01 mg. obtained in the original protective tests upon pigeons by Jansen and Donath. The agreement for *S. corallinus* is so close as to suggest more strongly even than before that vitamin B_1 is the *Streptothrix*-growth-promoting factor.

Table I.

A. *Pigeon tests.*

Bird	Month of test	Weight g.	Days on diet	Dose mg.	Day cure	Day dose mg.
(*a*) Doses introduced by the mouth:						
213	July	220	20	0·044	9	0·005
104	,,	217	19	,,	5	0·008
166	,,	240	17	,,	3	0·015
123	June	217	26	,,	5	0·009
174	July	250	25	,,	4	0·011
210	June	300	—	,,	11	0·004
153	,,	208	13	0·029	9	0·003
130	,,	224	20	0·087	8	0·011
149	,,	280	20	0·029	2	0·015
					Average	0·009 mg
(*b*) Doses injected into breast muscle in 0·5 cc. 0·9 % saline:						
103	Aug.	170	15	0·020	2	0·010
345	,,	250	30	0·018	7	0·0025
390	Sept.	294	24	0·024	4	0·006
254	Oct.	202	27	,,	2	0·012
411	,,	258	27	,,	6	0·004
365	,,	260	27	,,	4	0·006
					Average	0·007 mg.

B. *Rat tests.*

Rat	Dose mg.	Day cure	Day dose mg.
346	0·029	8	0·004
376	0·029	7	0·004
347	0·020	3	0·007
386	0·020	3·5	0·006
			0·005

C. Streptothrix *tests.*

This gave 0·006 mg. as the amount equivalent to 1 pigeon-day dose.

Summary

Method	Day dose mg.
Pigeon	0·009 (0·007 by injection)
Rat	0·005
Streptothrix	0·006
Average	0·007

Some estimate can be obtained from these figures of the amount required to promote growth in rats. Reader [1930] has shown that a young growing rat needs as a maximum 2·2 curative pigeon doses of vitamin B_1 daily to attain a maximal weight of 250 g. from 100 g.; less vitamin B_1 is needed to reach 100 g. It may be reckoned therefore that the maximum daily dose of

the crystals from rice polishings needed by the rat for growth to maximal weight does not exceed approximately 0·011 mg. daily[1].

Recently, Kinnersley and Peters [1930] stated that the Pauly reaction given by the purest vitamin B_1 preparations from yeast was a much less intense pink than that given by histidine, and that this seemed to constitute a difference between the Dutch preparation and their own. We have accordingly compared the intensity of the Pauly reaction given by one of the purest torulin preparations with an equivalent quantity of the rice vitamin side by side. The pinkness of the torulin preparation was rather greater than that of the rice vitamin; both gave a much less intense reaction than that of histidine. The difficulty therefore in the way of accepting the view that the same curative substance is present in the yeast preparation has disappeared. It must be concluded that the large doses required in the tests by Williams, Waterman and Gurin [1930] of 0·04 mg. *per diem* were a consequence of their method o testing.

SUMMARY.

Curative tests upon the Jansen and Donath rice vitamin crystals gave the following activity per day dose of vitamin: pigeon 0·007 mg. by injection, 0·009 mg. by mouth; rat 0·005 mg. Tests upon the micro-organism *S. corallinus* gave 0·006 mg. *per diem*.

We are indebted to the Medical Research Council for grants in aid of the above research and for personal grants to two of us.

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[1] (Note added at correction of proof.) The vitamin crystals also contain vitamin B_4 (see *Chem Ind.* (1930), **49**, *Proc. Biochem. Soc.* 990).

CCVII. THE ASSAY OF VITAMIN B_4.

By VERA READER.

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(*Received November 1st, 1930.*)

In earlier publications [Reader, 1929, 1930] evidence was presented for the division of the vitamin B complex into three components all necessary for the nutrition of the rat[1]. It was shown that two of these (B_1 and B_4) were alkali-labile while B_2 was not appreciably destroyed by autoclaving a crude yeast extract solution at p_H 9 for one hour at 120° [see Reader, 1930, p. 79].

The work reported here deals with the methods of assay used during the concentration and purification of the second alkali-labile factor B_4. In the earlier papers the restoration of growth of young rats, which had come to constant weight on a basal diet [Reader, 1929] plus excess of vitamins A, B_1, B_2 and D, was used as a criterion of the presence or absence of vitamin B_4 in a given extract. As there is a prolonged storage period for vitamin B_4 a certain amount of difficulty was experienced in keeping the animal colony sufficiently deficient in this vitamin, while, at the same time, supplying essential natural products (milk, etc.) to the breeding rats to get adequate lactation. Consequently a new "adult-curative" method was devised. Use of this method has led to the surprising discovery that the symptoms usually considered to be typical of polyneuritis in rats are really a mixture of symptoms due to lack of vitamins B_1 and B_4. By eliminating the vitamin B_1 deficiency at the critical stage the true clinical picture of vitamin B_4 deficiency is revealed and can be used in a curative test.

The main features of the adult-curative test are as follows. The animals, grown to maximum weight with adequate, but not excess, vitamin B_4 for normal growth, were completely deprived of both the alkali-labile vitamins B_1 and B_4 until polyneuritis occurred (3 to 4 weeks). At this stage it was found that vitamin B_1 cures the typical polyneuritis symptoms [see Kinnersley, Peters and Reader, 1930] but it does not restore weight or ameliorate all the symptoms, even when given in large excess doses daily, up to 12 pigeon doses per day. Instead the animal remains in a weak condition with swollen red paws, spastic gait, loss of co-ordination, and other symptoms apparently due to the specific lack of vitamin B_4.

Now if a positive test dose of vitamin B_4, proved free from vitamin B_1,

[1] The term B_4 is used throughout this paper to denote the third rat "B" factor, and is equivalent to B_3 in the earlier papers by the author. Thus it is hoped to avoid confusion with the Williams and Waterman thermolabile pigeon factor which has been termed B_3.

is given daily, the weight is immediately increased (20 g. per week) and at the end of 3 weeks all the above symptoms have disappeared (Fig. 1). If the substance contains no vitamin B_4, then the animal gets generally weaker and usually dies within 10 days, without any further marked loss of weight (Fig. 2).

Using this method it has been possible to concentrate vitamin B_4 from a 50 % alcohol extract of distillery yeast (British Drug Houses product) from 500 mg. to 0·4 mg. per daily rat dose, and to decide how far any given vitamin B_1 preparation is free from vitamin B_4.

Experimental.

Albino rats were brought to approximately maximum weight by feeding an approved basal diet [Reader, 1930] plus vitamins B_1, B_2 and B_4. It has been my experience throughout this work that it is necessary to give 2–3 pigeon day-doses of vitamin B_1 to each rat per day to get the normal growth rate (20 g./wk.). This is very interesting since it has been shown by Kinnersley, Peters and Reader that the curative dose for the polyneuritic rat is approximately the same as that for the pigeon. The vitamin B_2 was supplied as yeast extract autoclaved under the conditions previously described by the author [Reader, 1930]. The vitamin B_4 used for this earlier part of the experiment was always a stock preparation made from the decomposed mercuric sulphate precipitates obtained in the Kinnersley and Peters process for torulin (vitamin B_1). Thus the animals were brought to maximum weight under carefully controlled laboratory conditions.

These full-grown animals were deprived of the two alkali-labile factors (B_1 and B_4) for a period of about 4 weeks, or until polyneuritis occurred. During this period each animal loses nearly half its weight. Rather suddenly typical polyneuritis convulsions occur, accompanied by great weakness and ataxia. The paws are often oedematous and very red between the digits, and there is partial paralysis mainly manifested in the hind legs. Now the animal is given a dose of vitamin B_1 and the convulsions and paralysis disappear within a few hours. If daily doses of vitamin B_1 (2–3 curative doses daily) are now instituted, the animal can be maintained in this condition, *i.e.* with a general muscular weakness, spastic gait, swollen red paws, and a tendency to sit in a hunched position, for about 10 days. The weight, which has been falling rapidly up to this point, now ceases to fall, and maintains a steady level. If food and water are put at the far end of the cage, the rat can be persuaded to move, but frequently walks with a rolling movement and generally shows a lack of co-ordination. Such an animal is now ready for the vitamin B_4 test (see Fig. 1). The increase in weight is the first obvious sign that the sample under test contains B_4, but within 2 days the animal becomes much more active. At the end of a week the gait is apparently normal. If the sample tested does not contain vitamin B_4, the symptoms get rapidly worse and death occurs in 10–14 days.

When an animal has been completely restored to its original weight, it

may be used again for the same type of test. Frequently the same animal has been used four or five times, and it has been noted that for any given animal polyneuritis occurs each time at the same weight (± 5 g.) (see Fig. 2).

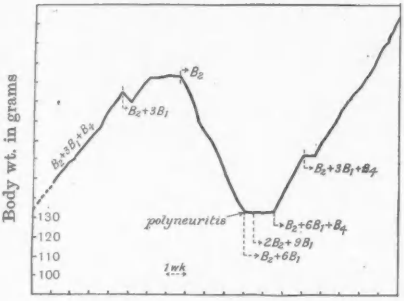

Fig. 1.

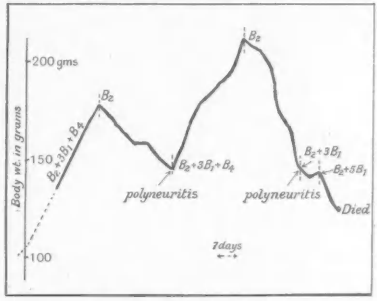

Fig. 2.

Figs. 1 and 2. Showing typical curves obtained when using adult rats for assay of B_4.

Discussion.

The technique elaborated above has one outstanding advantage that each animal is grown to maximum weight under carefully controlled conditions, and receives daily allowances of stock laboratory preparations of all three "B" factors. Thus any variation in the requirements of individual animals can be recorded and taken into consideration in the interpretation of later results. Although all the animals used in this work were bred in this department from an inbred Wistar albino strain, it is impossible to avoid occasional irregular results; probably the variations observed are not as great as with a mixed colony. An interesting observation is that an anomalous rat very seldom, if ever, requires excess of all three "B" factors, but rather appears to need a slightly larger dose of one of the several factors.

The symptoms described above have been frequently described, wholly or in part, in the literature. Originally they were thought to be due to a deficiency of vitamin B, then of B_1, and only now has it become evident that they are complex and due to a dual-deficiency of vitamins B_1 and B_4. Most workers have used young animals, and death may often have occurred before the complete development of all the symptoms reported in this paper.

In an accompanying paper [Kinnersley, Peters and Reader, 1930] it is shown how adult polyneuritic rats can be used for curative tests for B_1 and reference is made in this connection to Smith [1930]. In his table on p. 127, Smith shows a marked disparity in the ratio pigeon dose/rat dose for two preparations. From this he concludes that the maintenance test for pigeons (Seidell) is not a test for vitamin B_1 alone. However correct this conclusion may be, and there is evidence elsewhere to support it [Williams, Waterman and Gurin, 1929; Carter, Kinnersley and Peters, 1930], the experiments

described in this paper provide an alternative explanation of Smith's discrepancies. Either some of his litters may have been more deficient in vitamin B_4 than others; or, assuming that his rats were all equally deficient in vitamin B_4, the preparations under test may have varied in vitamin B_4 content. Until information is available upon these points, it cannot be decided whether his conclusions were justified. Since his preliminary period of depletion of 6–10 weeks is greater than the usual storage period for vitamin B_4, probably the problem of uneven storage does not arise. This 6–10 week period is worthy of comment since, with the adult albino rats used in this department, polyneuritis is always obtained in 21 to 28 days. It is still an open question how far the dual-deficiency of two vitamins hastens or hinders the onset of symptoms due to either one.

Sandels [1930] describes two types of polyneuritis, "acute" and "subacute or chronic" depending on whether the deficiency in the antineuritic vitamin is complete or only partial. Perhaps those of his animals which were subjected to a complete deficiency of antineuritic vitamin and died without showing noticeable symptoms of polyneuritis were suffering from a marked lack of vitamin B_4 also, especially as this often manifests itself in young animals as muscular weakness, followed by inanition and death. That his diets were lacking in vitamin B_4 may be judged from his description of symptoms, which include "cartwheel turning, spastic gait, and frantic circling when there was movement or noise in the laboratory." These I would not consider to be true polyneuritic symptoms, since it has been shown in the above experiments that they can be cured when, and only when, vitamin B_4 is supplied as a separate entity or in the vitamin B_1 extract used.

In conclusion, it must be emphasised that an animal will die from lack of vitamin B_4 even though a highly potent antineuritic concentrate (vitamin B_1) has been supplied daily in large excess for the 14 days preceding death (see Fig. 2). It is of interest, and perhaps of great importance, that all the concentrates of vitamin B_1, which have been obtained free from vitamin B_4, have been prepared from the $N/10$ HCl extracts of norite in the Kinnersley and Peters process for concentration of vitamin B_1. So far all the 50 % alcohol extracts from the same norite have contained abundant vitamin B_4.

At frequent intervals during the last 8 months parallel tests have been made on vitamin B_4 extracts by the adult-curative tests and the growth tests formerly described [Reader, 1930] and in all cases equivalent results have been obtained. The adult-curative method is now preferred since it is easier to control and gives a quicker result.

Appendix.

The following method for the concentration of vitamin B_4 has been used with much success when the precipitate to be decomposed is obtained by the application of the Kinnersley and Peters process to a B. D. H. product which we believe to be a 50 % alcohol extract of distillery yeast. So far the yield

has always been poor with the precipitate from the baker's yeast usually used in this Department. The reason for this is not yet clear.

Concentration of vitamin B_4. Wash the mercuric sulphate precipitate from 40 lbs. distillery yeast with distilled water until the washings give a blue colour with bromophenol blue. Suspend the precipitate in 6 litres water, pass H_2S for 4 hours, with occasional stirring. Filter, remove excess H_2S from the filtrate by distillation *in vacuo* (inside temp. 40–60°). At this stage there is danger of skin irritation due to unidentified volatile sulphides. Add NaOH to p_H 3, concentrate the whole *in vacuo* to 1 litre, adjust the p_H to 4·5 and add alcohol to 50 %. Allow to stand in cold store overnight, filter off any solid matter, remove alcohol *in vacuo* and concentrate to 400 cc. Add acetone to 80 %. Stir well and allow to stand at room temperature overnight. Decant next morning and remove acetone and some water *in vacuo* to 200 cc. (inside temp. never above 60°). Allow to stand in cold store for 2 days and filter.

The filtrate is now ready for dosing. The dose varies in different preparations from 1/20 to 1/4 cc.

To preserve, adjust the p_H to 4 and add alcohol to 20 %.

SUMMARY.

An adult-curative method for the assay of vitamin B_4 is described.

My thanks are due to Prof. R. A. Peters for his advice and criticism throughout the work, to him and Mr Kinnersley for numerous samples of concentrates for testing and for checking my own preparations by tests on polyneuritic pigeons, and to Miss M. Kempson for help with the animals; also to the Medical Research Council for a personal grant.

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CCVIII. MAINTENANCE NUTRITION IN THE ADULT PIGEON, AND ITS RELATION TO TORULIN (VITAMIN B_1). I.

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(*Received October 23rd, 1930.*)

Our interest in this subject arose from the desire to meet possible objections to our original contention that a curative and protective vitamin B_1 preparation would not give maintenance nutrition [Kinnersley and Peters, 1925]. We found it impossible to effect this completely without a more elaborate investigation (of which this paper forms part) into the factors needed to give maintenance nutrition in the normal pigeon. Accurate knowledge upon this is essential for the use of weight maintenance tests with pigeons in the assay of vitamin B_1 and also for a study of the symptoms of avitaminosis. As pigeons upon a polished rice diet are suffering, in addition to deficiencies of protein and salts, from a deficiency of more than one B factor, the pathology of avian polyneuritis cannot be properly understood until single instead of multiple deficiencies can be experimentally produced [Peters, 1929, 1930]. We need not stress the wider consideration that a full knowledge of the necessary factors, as they present themselves in various animals, seems to be fundamental to the study of human beri-beri. In this paper, B_1 and B_2 have their accepted meaning. The term B_3 is used for the pigeon factor [Williams and Waterman, 1928] and B_4 for the rat factor [Reader, 1929].

Historical.

A survey of the literature upon vitamin B shows much disagreement as to whether polished rice with addition of vitamin concentrates is sufficient to constitute a complete diet. Schaumann [1914] for instance said that this was not so. We find in the earlier work that there were attempts to compare the curative activity of vitamin B extracts with their powers of maintaining weight in the adult pigeon. An especially valuable study in this connection was made by Cooper [1913], who graded certain foodstuffs according to their power to prevent polyneuritis, and then found that this grading did not hold for their capacity to protect from loss of weight. Such evidence was very suggestive of two factors, but could not be taken to prove this, because it could be interpreted in other ways, as *e.g.* by the presence of inhibitory substances in some

of the foods tested. Somewhat later Funk [1914] concluded that polished rice plus vitamin B_1 (a preparation of about 1 mg. per day activity) constituted a complete diet, though this conclusion does not stand out firmly, as he alludes in the same paper to another preparation which he obtained from yeast, which was curative but not effective in complete nutrition. Williams and Seidell [1916] found that the curative and weight-maintaining powers of vitamin B concentrates could be separated, but were inclined then to attribute this to the decomposition of the vitamin. Emmett and McKim [1917] showed clearly that supplements of vitamin adsorbed on fuller's earth and Lloyd's reagent would induce a rise in weight if given in sufficiently large doses, but that there was a quantitative difference between the amounts required to cure and to give full nutrition, when the original yeast extract was compared with the concentrate [see also Chick and Hume, 1917]. Kellaway [1921] found that polished rice with a daily addition of 1·0 g. of marmite led to an average daily gain of 1·7 g. over a period of 1 month, additions of 2·0 g. did not increase this gain, whereas 0·75 g. under the same conditions led to a gain of only 0·5 g. *per diem*. Plimmer and Rosedale [1923] found that 3·0 g. marmite to 90 g. rice + 15 g. fish meal gave maintenance of weight in some birds. Tsukiye [1922] using a vitamin concentrate from rice polishings found that it caused a gain in weight in hens feeding upon polished rice. Dissatisfaction with pigeon curative tests led to search for a new technique and the simple problem as to whether the vitamin was the only supplement needed to complete a polished rice diet was begged by the use of a weight maintenance test for vitamin B, which was introduced by Seidell [1917, 1922]. In this test, pigeons fed upon polished rice are given daily doses of vitamin B concentrate, and the amount of vitamin is gauged by use of the fact that weight is maintained practically constant if sufficient is present. This method was also adopted by Funk and Paton [1922] and by R. R. Williams, Eddy and their co-workers. Its simplicity as compared with the curative tests led to a rather wide adoption and to the belief that a substance could only be considered to be vitamin B if it were capable of preventing polyneuritis as well as inducing maintenance of weight upon a polished rice diet in pigeons. This conclusion was reached, it must be noted, in spite of the observation by Seidell [1921] that certain silver-baryta fractions were potent as curative but not as weight-maintaining substances. Abderhalden and Schaumann [1918], on the other hand, seem never to have accepted the view that the curative and weight maintenance factors were identical [see also Berg, 1923].

In 1925, after two of us had demonstrated to our satisfaction that the curative substances as such could be fractionated and concentrated to a high activity, we used up the remainder of the preparation then in our hands in an attempt to induce with it maintenance nutrition in pigeons and prophylaxis against polyneuritis. In this way it was found that two curative day doses of torulin (vitamin B_1) administered daily to pigeons upon a polished rice diet did not protect against loss of weight, though they protected against poly-

neuritis for over 30 days. Larger doses of the same preparation however after considerable loss of weight had occurred gave in one case a weight maintenance, but without storage of the vitamin for any dose other than the last dose administered. In so far as the vitamin concentrates of Seidell [1917, etc.], Funk, Harrow and Paton [1923], etc., maintained weight when given as supplements to polished rice, we drew the conclusion that more than one factor must be needed, and that vitamin B must be dual. Our experiments did not satisfy us completely. We had relied upon the statements of others that yeast extracts would support a maintenance nutrition. This might not have held under our conditions. Further there was always the question as to whether we had administered enough for maintenance. It was possible that more than twice the curative dose should be given each day to induce maintenance. As soon as larger amounts of the more active torulin preparations were available, we returned to the question. Jansen and Donath [1926] showed that their rice vitamin B would support a weight maintenance in pigeons only when supplemented with $2\frac{1}{2}$ % of meat powder daily and cod-liver oil; in this case they considered that the meat powder was supplying a deficiency of protein. The idea that the meat supplied some missing protein factor did not agree with previous work, because whatever was the supplement needed in the meat must have been present also in Seidell's vitamin B concentrates. Williams and Waterman [1928] described a factor (vitamin B_3) necessary for pigeons to gain maximum weight, as distinct from maintenance at some intermediate weight. This was not present in certain antineuritic yeast concentrates, though it was present in whole yeast and in whole wheat. These facts have been confirmed: marmite, a yeast preparation, containing vitamins B_1, B_2 and the Reader rat factor B_4 [Reader, 1929], will not induce more than a maintenance nutrition even when supplemented with caseinogen and salts; but rise to maximum weight takes place readily upon giving whole wheat [Peters, 1929]. In the Harben lectures [Peters, 1929], we stated that 3 day doses of a torulin preparation, given daily from the start of a feeding, did not induce maintenance of weight at any stage of nutrition. The fact that marmite gave maintenance and did not appear to contain the Williams and Waterman vitamin B_3, we explained by postulating the need of a supplement to vitamin B_1 for maintenance. But later Guha [1929] stated that adult pigeons upon vitamin B_1 preparations from wheat germ and brewer's yeast showed maintenance, or nearly so, with doses equivalent to 5–6 curative pigeon doses daily. Our proof therefore did not really cover this point, though the three doses given would have been for many pigeons equal to 5 (we chose the lowest figure given by our birds). This matter has now been settled. A preliminary account of our work has appeared [1930], and at the same time there came into our hands through the courtesy of Prof. Eddy the papers by Eddy, Gurin and Keresztesy [1930], and by Williams, Waterman and Gurin [1930]. In the latter paper the authors show that the Jansen and Donath vitamin is protective to pigeons against polyneuritis, but does not give maintenance nutrition. They have therefore

reached by a different route essentially the same conclusions as ourselves, though the fact that their preparations are not standardised in the same way makes it difficult to decide whether the point raised by Guha has been covered in their work.

In order to prove that vitamin B_1 does not alone give maintenance in pigeons feeding upon polished rice, it must be shown that an amount of vitamin B_1 adequate for maintenance in an impure yeast extract does not suffice when a purer vitamin is given instead. So as to make certain that variations in birds are not influencing results, the purer vitamin B_1 must be given in excess of the largest amount which normal birds require. In our case all the extracts have been standardised by the same method, and we have used as a basis a standard preparation of crude marmite. Our experiments have been planned as simply as possible in order to narrow the issues. Polished rice has been supplemented with the crude yeast extract: we have then tried to imitate the effect of this crude yeast extract by various torulin preparations. We shall first describe what we mean by maintenance nutrition, and then show in confirmation of Kellaway [1921] that a supplement of approximately 1·0 g. of marmite will produce maintenance for quite long periods. There will then follow the evidence that large amounts of yeast vitamin B_1 do not imitate marmite as supplement; this is a rigid proof of our former point, and shows that vitamin B concentrates which induce weight maintenance (such as that of Seidell) must be composite. The next question arising is how much vitamin B_1 is wanted to give weight maintenance. This involves a knowledge of the other factors supplementing B_1, and has not yet been settled.

Experimental.

Over 100 pigeons have been used during the course of this research. We have gained much from a continuous study of 15 pigeons over a period of 20 months. The birds have been kept upon screened bottom cages in groups in a room of temperature varying between 10 and 18°. We have run control experiments, but have not been able to find that variation in temperature affected the results. Pigeons have been fed upon polished rice and tap water with doses every third day of cod-liver oil. The polished rice has been treated by our usual methods. Birds have been weighed at 9.30 a.m. and doses of curative extract given into the crop at 4 p.m. Where more than one extract had to be dosed to the same bird, the extracts were given separately. In all cases the birds have been allowed to feed naturally, and no attempt has been made to check the amounts of food eaten. In spite of the rather sweeping remarks of Mitchell [1929] upon this point the amounts of food do not concern our facts as to weight and its maintenance.

Standardisation of the curative extracts.

Since the proof below depends ultimately upon the methods, these require discussion. That of Kinnersley, Peters and Reader [1928] has been used

throughout, and the average figures for the preparations are given below. We have given the outside figures of the deviation from the mean in the case of the marmite and of the X preparation, so that we can exclude the possibility that the figures upon which reliance is placed in this paper are vitiated by the selection of birds which lay upon the outside limit of our averages.

Marmite. This has been used as a standard in the form of the crude extract supplied by the company. The curative figures for 1·0 g. marmite were in days, 4, 3, 4, 4, 4, 6, 3, 7, 4, 6, 3, 4, Av. 4·3. The significant figures are the average 4·3, and the maximum of 7. This it will be seen is quite exceptional.

Torulin. It has been our custom [Kinnersley and Peters, 1928] to make two extractions of the charcoal, one by aqueous HCl (X preparation) and the other by alcoholic HCl (Y preparation). The X preparation is purer, and as we shall show has different properties.

X preparation. Out of a series of birds this reacted very constantly at 4·0 days per 0·1 cc. One bird gave 3 days.

Y preparation. The figures here given for the average of 3·0 were the average of 37 tests. It has been our experience that the tests with this preparation are not so consistent as those with others. We do not profess to understand this at present, but merely record it as our impression that the deviation from the mean curative dose is greater with some preparations than with others.

Methods of preparing the concentrates used in these experiments.

X preparations. *N*/10 HCl extract of the charcoal. This material was obtained from 112 lbs. of baker's yeast, by the method of Kinnersley and Peters [1927, p. 785]. After boiling off H_2S and reducing to a volume of 40 cc., the solution was fractionated with alcohol by the method of the same authors [1928, p. 426], the fractionation being carried to the absolute alcohol stage, with a change of reaction to p_H 3·5. After removing the alcohol, the residue of approx. 4·0 g. was dissolved in 150 cc. water, and sufficient HCl added to make acid to Congo red. It was then treated with 0·5 g. norite charcoal. The charcoal was separated and extracted with *N*/10 HCl to remove any adherent vitamin. To the combined filtrates, 115 cc. of phosphotungstic acid (10 % in 10 % H_2SO_4) were added, this amount being short of that required to produce maximum precipitation. The phosphotungstate was decomposed by grinding with solid baryta, excess Ba removed from the filtrate with sulphuric acid, and the reaction brought to Congo red acidity with HCl; NaOH was then added to p_H 3·5–4·0, and the solution concentrated first on the water-bath and then *in vacuo* at a low temperature. It was then fractionated until soluble in 99 % alcohol, giving torulin, which remained stable in alcoholic solution for over 2 years, of an activity rather greater than 0·1 mg. per day.

Y preparations, prepared from the 50 % acid alcohol extract of the charcoal. Two of these were used, A and B. (A) was made from baker's yeast by our usual methods; (B) was a mixture of a preparation from baker's yeast,

and of one from a British Drug Houses concentrate. They were both fractionated by a new phosphotungstic method, shortly to be described in detail. In view of the lengthy description which would be entailed if all the experimental details were given in the latter case, we propose to make only the essential points clear[1].

(A) Preparation from 56 lbs. of baker's yeast. The details described by Kinnersley and Peters [1927, pp. 785, 786] were adhered to with the following exceptions. The yeast was extracted upon arrival at the laboratory. At the baryta stage, a small volume of hot baryta solution was used to precipitate the gum, instead of the large volume of cold solution. We have used this modification of the original process now for some time, as we have found that it greatly diminishes the volume at the final stage, and therefore facilitates the manipulations.

After extracting the charcoal with $N/10$ HCl, the 50 % acid alcohol extract was made as usual. The acid alcohol extract of 3 litres was concentrated to 200 cc. on the water-bath after addition of NaOH to make purple to Congo red. Water was then added to 400 cc., the solution brought to p_H 3·5 and H_2S passed for 3 hours. The sulphide was suspended in acid water and H_2S again passed. The p_H of the combined filtrates was brought to 2·5 approximately, and the H_2S removed by boiling. To the volume of 460 cc., 70 cc. absolute alcohol were added and HCl to p_H 2·0 (turn of thymol blue). The extract was kept in cold store. Activity 3·1 mg., 6300 doses.

(B) This was a mixture of a preparation made from (1) 51 lbs. of baker's yeast and (2) B.D.H. concentrate. (1) was carried out to the charcoal stage by the usual methods with the exception that the yeast was allowed to stand for 12 hours in 16 litres of water before extraction. After adsorption upon the charcoal, the stage of $N/10$ HCl extraction was omitted. A 50 % alcohol acid extract was made, concentrated, and then fractionated twice by our newer phosphotungstic methods, and finally with alcohol. Activity 0·16 mg., 2900 doses. (2) Concentrate purchased from British Drug Houses. This concentrate is said to be from distiller's yeast. As supplied it is stated that 1·0 g. equals some 20 g. of the original yeast. As we have not yet given the details of working up this material, as far as the charcoal stage, we shall give these in greater detail. The main advantages are that work can be carried out with smaller volumes than when the initial step is the yeast itself, and the laborious stage of extraction is avoided. Against this has to be set the disadvantage that the commercial preparations are apt to be variable in activity.

5 lbs. of the concentrate (equal to 90 lbs. of the original yeast) were diluted to 24 litres. 2800 cc. lead acetate (25 % neutral) were added, and the precipitate was removed by filtration. The filtrate was then treated with sufficient hot baryta to precipitate the gum (this step being the same as in the case of the ordinary preparations), and the excess barium removed with sulphuric acid. The solution was brought to p_H 3·0 and 1100 cc. 10 % mercuric sulphate

[1] Should the experimental details be needed, we shall be happy to supply them.

solution (Hopkins) stirred into it, the large precipitate being removed by filtration. The filtrate was brought to p_H 7·0 and treated, in all, with 900 g. of norite charcoal. Two extractions of the charcoal were made first with $N/10$ HCl and then with acid alcohol. In the particular case under consideration the $N/10$ HCl stage was omitted, the charcoal being extracted five times with 50 % acid alcohol, about 6000 cc. being used. The extract was concentrated to 1060 cc. This was treated with 5·0 g. of charcoal, and the filtrate extracted three times with ether. The ether was removed upon the water-bath, volume 400–500 cc. At this stage it was fractionated twice with phosphotungstic acid by the new methods. When combined the above fractions gave 6000 day doses of torulin (approx. 0·2 mg./day).

Maintenance nutrition.

Several nutritional conditions can be recognised in pigeons. There is a weight minimum which is about 60 % of the weight maximum. There is also a rising, falling and maintenance nutrition, the terms being self-explanatory. Weight maintenance may be defined for our purposes as a condition in which the weight remains constant over a period of 30 or more days, with a continuous daily rise or fall of not more than 1·0 g. Work upon this problem has convinced us that it is necessary to distinguish these conditions as in some sense separate nutritional states. For instance, it requires less of a yeast concentrate to keep a bird at a weight near the weight minimum than at other weights. There is another possible complication, which we wish to exclude from discussion in this paper. It has been our experience in dealing with pigeons for curative tests that some difference in the bird appears after feeding upon polished rice for more than 30–35 days. Others seem to have felt this too, because they have also chosen birds for test within this approximate period. In order to narrow the issue here, we propose to limit our attention to pigeons which have been nourished upon the following diets within 35 days of the termination of the experiment, namely whole wheat, mixed grain, or polished rice supplemented with marmite.

In our experience the weight maintenance, which is so characteristic a feature of the work of Seidell and others, can be produced at most stages of nutrition between maximum and minimum in pigeons feeding naturally upon polished rice by adding to the diet doses of marmite given by tube into the crop. This is a remarkable happening from a physiological standpoint. Fig. 1 is typical of such a maintenance at intermediate nutrition. It shows the preliminary fall upon polished rice, the maintenance with an addition of 2·0 g. of marmite autoclaved at 115° for 1 hour (A), and the fall when the temperature of autoclaving was raised to 120° (B). In confirmation of Kellaway [1921], 1·0 g. of fresh marmite daily is sufficient in most pigeons to give maintenance. Kellaway found a slight rise, but this may well have been due to differences in the marmite preparation. Fig. 2 shows the effect of increasing the amount of marmite from 0·75 g. *per diem* up to 1·0 g., 2·0 g. and finally

4·0 g. A marked rise in weight takes place which tends to become stationary, and is never the equivalent of that obtained with whole wheat; the weight stops short much below the possible maximum. It is probable (see Part II) that these rises in weight are induced by traces of vitamin B_3 still present in the marmite.

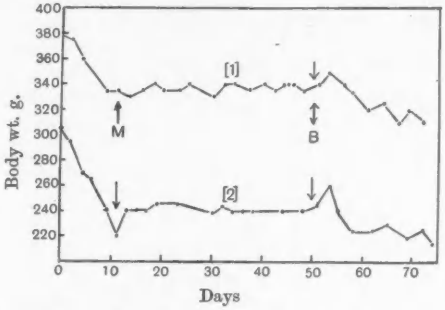

Fig. 1. Maintenance on marmite. Doses of marmite, autoclaved at 115° given at *M*. At *B*, diet changed to salted rice and marmite autoclaved at 120°.

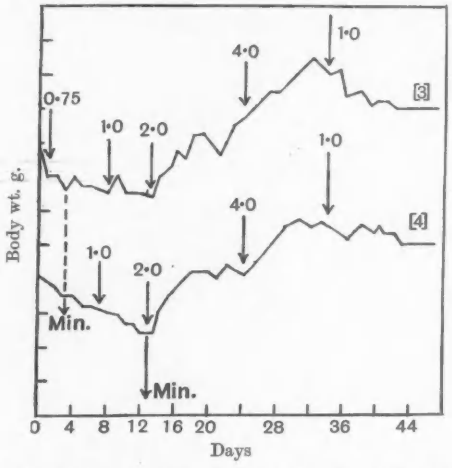

Fig. 2. Large doses of marmite. Ordinates. Divisions 20 g. Weight on 20th day, bird 3, 420 g.; bird 4, 385 g. Figures at arrows refer to daily doses of marmite in g. Minimum possible weight for bird 3 marked by dotted arrow.

Accepting 1·0 g. of our marmite preparation as the maintenance dose at an intermediate weight, we have found that such an amount contains 4·3 curative pigeon day doses. If vitamin B_1 is the only effective supplement in the marmite, then this amount of our concentrates standardised under the same conditions should imitate the dose of marmite. On the contrary,

reference to Fig. 3 shows that 8–12 day doses of some of our concentrates (X) are not enough to give maintenance[1]. They are not even enough if we assume that in the marmite experiments we had selected the most sensitive birds, and in the others the least sensitive. If we had done this the figures would be 7 doses for marmite and 9 doses for the X preparation. In bird 6, transference to 1·0 g. marmite on 38th day readily gave maintenance. It is therefore proved that vitamin B_1 alone is not adequate for weight maintenance, and thus our former point is firmly established. In this matter, we find ourselves to be in complete agreement with the latest work of Williams, Waterman and Gurin [1930], who have used the Jansen and Donath vitamin from rice together with a protective test.

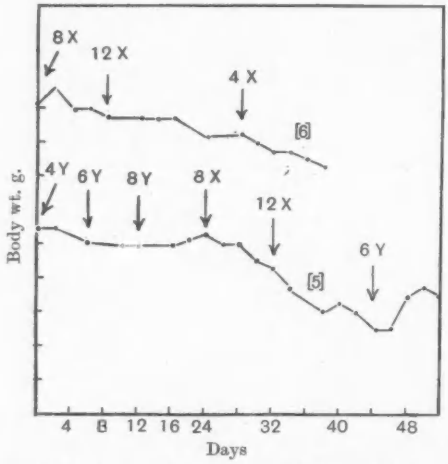

Fig. 3. Doses of vitamin B_1. Ordinates. Divisions 20 g. Weight on 24th day, bird 5, 375 g.; bird 6, 375 g. Numbers upon charts represent the number of vitamin B_1 doses of X or Y preparations.

How then are we to explain the results of Seidell [1926] and of Guha [1929]? We find that maintenance is produced by giving 6–8 doses of our 50 % alcoholic extracts of the charcoal (Y concentrates) (Fig. 3). Bird 5 illustrates this. These imitate the effect of the marmite. As little as 1·2 mg. of our purified Y preparation of vitamin B (YB) was sufficient to give weight maintenance. With this preparation there was a tendency for the weight to drop in some birds towards the 30th day of feeding. Since this "YB" was effective in such small amounts, and had been purified by phosphotungstic acid in addition to the charcoal adsorption, it is evident that the maintenance

[1] Some X preparations are not so free from the other factor. Out of all the birds and the various X preparations tested, we have never found a case in which 4–6 day doses were sufficient for maintenance. With 10–12 doses in the case of one bird upon one of the X preparations, the drop was not larger than 1·0 g. *per diem*. Even this was in contrast to similar doses of a Y preparation, which gave no loss or a slight increase in weight.

effect produced by marmite (in contradistinction to the fall with vitamin B_1 alone) is not due to the presence in the marmite of necessary protein or salt factors in the ordinary sense. The Y concentrate therefore contains some factor (or factors) in addition to vitamin B_1 needed by the pigeon for maintenance. It follows from Seidell's experiments and from our own that this factor tends to accompany vitamin B_1 in the processes of purification. Jansen and Donath [1926] found that their rice vitamin did not give weight maintenance in pigeons unless it was supplemented with an addition, which could be supplied in their case by meat powder thoroughly extracted with boiling water. They considered that this was a supply of protein. Our experiments with the Y preparation show that the factor which they were supplying was not protein in the ordinary sense, but one which can also be concentrated from an aqueous extract of baker's yeast. It is clear that we cannot settle how much vitamin B_1 a pigeon needs for maintenance until we can supply all other B factors in excess, and hence until any supplementary factors can be isolated. In the Harben lectures, we suggested that the factor needed as supplement might prove to be identical with vitamin B_4 which is known to be present in the Y type preparations[1]. Subsequent work which is being prepared for publication suggests that the supplement involved differs from previously characterised factors of the vitamin B complex.

Discussion.

This paper settles part of our original problem. Vitamin B_1 (from yeast) will not give maintenance nutrition in pigeons unless it is supplemented with some undefined factor. It is obvious, as pointed out by Williams, Waterman and Gurin [1930], that work upon vitamin B_1 in which maintenance tests upon pigeons have been used must be substantially revised. The contention [Kinnersley, Peters and Reader, 1928] that experiments such as those of Plimmer *et al.* [1927] must be interpreted as experiments upon the vitamin B complex is certainly justified. Until some chemical test is available, we are inclined to think that curative tests upon the pigeon or the rat [Kinnersley, Peters, and Reader, 1930; Smith, 1930] are our only reliable means of standardising foodstuffs for vitamin B_1 in spite of the rather large variations shown by such tests. The protective test is complicated by two considerations.

(*a*) It implies a knowledge of the time of onset of symptoms in the absence of vitamin B_1. In our experience this is a variable quantity in different pigeons. It might be possible to make use of our finding that individual pigeons show a surprising uniformity in the time at which symptoms appear. So far as we know, this has not yet been done.

(*b*) The second consideration is rather more serious. We have pointed out elsewhere (Harben lectures) that after the 30th day of rice feeding, pigeons appear to need more vitamin B_1, because 3–4 day doses of torulin given daily

[1] Reader (personal communication).

were not always sufficient to protect against symptoms. In their recent assay of the Jansen and Donath rice vitamin, Williams, Waterman and Gurin [1930] obtained a figure of 0·04 mg., a higher figure than that of the original authors' tests of 0·01 mg. *per diem*. We think that this is possibly due to the fact that they made preventive tests at the weight minimum, upon pigeons in the period after the 30th day of rice feeding.

We have refrained from discussing the most interesting feature of experiments of this type, the fact that these minute additions to the diet can enable the animals to balance intake and output so that weight is maintained for long periods within a few grams, at a point intermediate between the maximum and minimum weight of which the animals' economy is capable. A physiological adjustment is surely here indicated as precise as those which Haldane and others have shown to regulate the breathing.

Summary.

1. In adult pigeons feeding naturally upon whole wheat or polished rice, with or without supplements of marmite or torulin concentrates, there can be distinguished a maximum, minimum or intermediate weight, and a rising, falling or maintenance nutrition.

2. A daily dose of marmite of 1·0 g. is sufficient to convert a falling nutrition upon polished rice into maintenance at almost any point between the maximum and minimum weight. This dose of marmite contains 4·3 day doses of vitamin B_1.

3. Large amounts of vitamin B_1 (up to 12 doses) given as the $N/10$ HCl extracts of the charcoal do not imitate the effect of the marmite. This establishes our former contention that purified torulin (vitamin B_1) does not give maintenance.

4. Maintenance nutrition is however given by 6–8 doses of our 50 % alcoholic extracts of the charcoal. These therefore contain some other factor necessary for avian nutrition.

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CCIX. MAINTENANCE NUTRITION IN THE PIGEON. II.

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In Part I [1930] it was proved that purified torulin would not give maintenance nutrition unless supplemented with some factor present in alcoholic extracts of the charcoal (Y preparations of vitamin B_1, Kinnersley and Peters process), and also in marmite. This factor is probably present in Jansen and Donath's meat powder, but absent from, or much reduced in, the $N/10$ HCl extracts of the charcoal (X preparations). We wish to present the evidence that the factor in question differs from the pigeon factor B_3 [Williams and Waterman, 1928] and that at least three B factors are therefore required by the bird for full nutrition. This establishes more firmly the conclusion put forward previously [Carter, Kinnersley and Peters, 1930]. We shall also show[1] that the unknown factor differs from the characterised rat factors B_2 and B_4. Before considering the evidence relating to specific factors, some general considerations require discussion. It is evident that a bird will not regain maximum weight from any lower weight unless all necessary factors are supplied. The simple assumption guiding our previous work, that, if a diet is complete except for three factors, the presence of any two of them will give maintenance, and the supply of the third rising nutrition, cannot be supported in theory. We are inclined to think now that it cannot be supported in fact. From a theoretical standpoint, it is likely that an animal will not maintain weight unless traces of all necessary factors are present; the limiting factor would determine the stage at which the weight was stabilised. This proposition cannot be proved until concentrates of all the necessary factors exist proved to be free from other factors, and until we have full knowledge of the storage capacity of birds at various seasons of the year for the various factors. Further work has indicated that contrary to our previous statement, marmite must contain vitamin B_3 in small amount. If sufficient marmite[2] be given, especially together with X and Y preparations, pigeons may gain in weight at the rate of 5·0 g. *per diem*. The maintenance produced by 1·0 g. marmite may therefore be the result of two factors in excess operating in the presence of a trace of a third factor. Alternatively, the two factors present might cooperate with traces of

[1] Preliminary communication (1930). *Chem. Ind.* **49**, *Proc. Biochem. Soc.* p. 517.

[2] In our experience, marmite has given consistent results.

the third factor stored by the bird. That this alternative can influence results is shown very clearly in Fig. 1. Bird 1, whose weight had been stabilised by

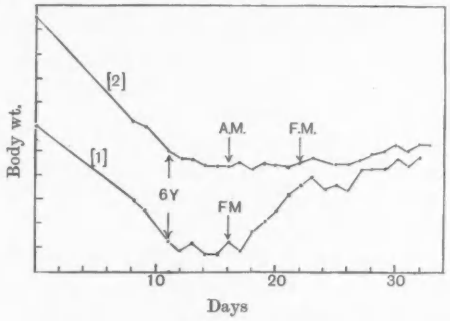

Fig. 1. Storage effects. Ordinates. Each division 20 g. Body weight on 30th day; bird 1, 355 g.; bird 2, 345 g. 6 Y means 6 doses daily of vitamin B_1 as Y preparation. A.M. means 1·5 g. daily alkalised marmite. F.M. means 1·5 g. daily fresh marmite.

dosing daily from the 11th day with 6 doses of YB, was given in addition on the 16th day 1·5 g. daily of fresh marmite. It will be noticed that the gain in weight is sudden and large, at the rate of 10 g. a day for 6 days[1]. Similar rises, though not quite so large, have been observed in birds dosed similarly with marmite on the 18th day after putting upon polished rice. That the effect is due to storage, is shown by the chart for pigeon 2, which was a control experiment. Alkalised marmite was given upon the 16th day, instead of fresh marmite, for 6 days, after which fresh marmite was substituted. Here the gain in weight did not take place for several days, after which there was a slow continuous rise. This slow rise will continue at the rate of 3·0–5·0 g. *per diem* up to the 40th day and longer and shows that traces of all necessary factors are present in marmite, though not sufficient to give maximal increases. The effect shown in Fig. 1 must be a storage effect and due to the addition of the traces of vitamin B_3 in the marmite to that stored by the bird. In the paper upon vitamin B_3 recently published by Eddy, Gurin and Keresztesy [1930], conclusions as to the presence or absence of vitamin B_3 are drawn from gains in weight varying from 1·0 to 5·0 g. *per diem*. No mention is made as to the existence of these storage effects, and we do not know therefore whether their possible presence has influenced any of their conclusions. They form an explanation of a puzzling feature of the composite curve recently published [Peters, 1930; Harben lectures II, Fig. 5, A]. The curve showed a sharp rise for a matter of 10 days after giving marmite and then again fell. The effect was not given by all birds and is clearly due to storage. A late recognition of these facts has led us to adopt certain principles. Conclusions as to absence or insufficiency of factors may be legitimately drawn from a falling nutrition at almost any stage after commencing a rice feeding, provided that the weight

[1] On whole wheat, birds gain weight more rapidly than this.

is still above 65–70 % of the weight maximum; conclusions as to the presence of factors in the preparations under test may only be drawn from a maintenance nutrition, when there was clear previous evidence of falling nutrition, or when the period of rice feeding has extended over at least 20 days. We have taken pains to conform to these principles in this paper. Fig. 1 indicates a point of great interest. It has often been pointed out that rice constitutes an inadequate diet because of its inadequate protein. In spite of this, the addition of marmite in the case of pigeon 1 was enough to enable a gain in weight of 10 g. a day to take place. Provided that the B factors are correct, we have not obtained experimental evidence for the view that the polished rice is inadequate for maintenance of birds in the short periods (up to 40 days) under consideration. We showed previously that maintenance of birds upon polished rice with additions of 2·0 g. of marmite a day was not affected by the giving of caseinogen (1·0 g. *per diem*). Hence caseinogen could not induce the rising nutrition which is seen when the rice is supplemented with Y preparations and marmite. However contrary to accepted principles this may seem to be, we believe that it has experimental foundation.

Accepting the fact that vitamin B_3 is necessary in pigeon nutrition, in order to show that three factors at least are required, evidence must be given to establish the difference of the unknown supplement from vitamin B_3.

Vitamin B_3. Substances have been regarded as containing B_3, if they would induce rather rapid gains in weight up to the maximum possible, either from an intermediate weight, or from a minimum weight [Williams and Waterman, 1928] in pigeons feeding upon polished rice with daily supplements of vitamin B_1 in the form of either a fuller's earth concentrate, or concentrates stated to be prepared by our methods. A further property of vitamin B_3 upon which stress has been laid is its thermolability, especially after treatment with alkali. Eddy, Gurin and Keresztesy [1930] say that after alkaline treatment vitamin B_3 disappears even at 20°. Upon these grounds we were led originally to reject absolutely the possibility that our supplement was vitamin B_3 or that marmite contained this vitamin. The evidence below that alkalised marmite contains the unknown factor certainly is hard to reconcile with the above properties. We are however well aware that the stability of B factors is often determined by the mixtures in which they find themselves, and have had good examples of this already in the case of Vitamin B_1 [Kinnersley and Peters, 1928]. There is a possible indication that the thermolability of vitamin B_3 is a question to some extent of conditions. Guha [1929] reported that extracts of vitamin B_3 could be prepared by extracting brewer's yeast with hot water. We have found that the vitamin B_3 in whole wheat resists steaming to some extent. Our experiments were originally planned in the hope of obtaining a basal diet other than rice, free from vitamins B_3 and B_4. The results were unexpected. Birds upon wheat steamed for 2 hours do not lose weight even when at the nutritional maximum. When the wheat has been steamed for 5 hours, the weight was lost as if the birds were upon polished

rice. Fig. 2, bird 3, shows the weight curve of a typical bird upon wheat steamed for 5 hours to which from the 15th day additions of vitamin B_1 were

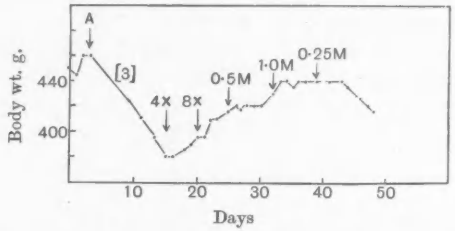

Fig. 2. Effect of steamed whole wheat. A refers to start of wheat feeding. Numbers refer to doses of vitamin B_1 as X preparation or to g. marmite.

made. At first we assumed that the loss of weight was due to the destruction of vitamin B_3 which we had believed to be thermolabile (as suggested by Williams and Waterman). There is however a destruction of vitamin B_1, because many birds get polyneuritis, after some 20 days upon this diet, the polyneuritis being cured by doses of vitamin B_1 by the mouth. The loss of vitamin B_1 is not due to enzyme processes, because wheat kept at 40° in a steamer for 2 hours behaved as fresh whole wheat. It is possible that it is due to inhibitory toxic factors formed in the steaming. A fact of interest is that quite small supplements of marmite, certainly not more than 0·5 g. (= 2·1 doses of vitamin B_1) are enough to produce rising nutrition; a similar effect has been produced by giving not more than four B_1 doses of an X preparation (see Fig. 2). Since rising nutrition must require the presence of vitamin B_3, it might be concluded that both vitamin B_3 and our postulated maintenance supplement are more stable to heat under these conditions than vitamin B_1. Hence this raises the question as to whether vitamin B_3 is still present in the Y preparations, and could therefore be the unknown supplement.

We have stated previously that YA in doses of 8 B_1 *per diem* did not cause a rising nutrition at intermediate weight, but gave maintenance. If vitamin B_3 is present at all in Y, then large increases of this should give some gain in weight. Fig. 3 shows the results of giving enormous doses of YB, up to 36 doses *per diem* to birds upon polished rice diet. There was a slow rise in weight which did not persist in bird 4 but did in bird 5.

Further the birds at the 35th day were still 100 and 70 g. respectively below maximum weight. They were quite normal. The 1·5 g. alkalised marmite given for 3 days from the 21st day does not seem to have had any certain effect. The experiment is interesting apart from the present connection, because it is the first time that such large doses of vitamin B_1 have been administered to animals. In the present connection it indicates that there may be small amounts of vitamin B_3 present in the Y preparation: whereas 6 doses are enough to give maintenance in most birds, 36 doses do not induce more than

small rises in weight. Now slight increases in vitamin B_1 supplementing a vitamin B_1-deficient whole wheat diet convert to rising nutrition; the case is not apparently different for vitamin B_3. Examination of the tables published by Eddy, Gurin and Keresztesy [1930] show a case in which a falling nutrition upon 0·75 g. of yeast was converted to a rising one by increasing the yeast to 1·0 g. For the known factors therefore there is no evidence that change from maintenance to rising nutrition requires large increases of a B factor: there is no reason why it should be postulated for vitamin B_3.

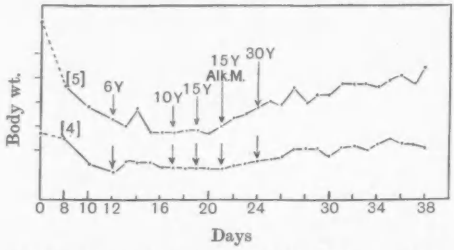

Fig. 3. Large doses of vitamin B_1. Ordinates. Each division 20 g. Body weight on 20th day; bird 4, 345 g.; bird 5, 315 g. Numbers refer to number of doses of vitamin B_1 as Y preparation given daily. Alk. M. means 1·5 g. alkalised marmite.

Hence there are three lines of evidence to show that the unknown is not vitamin B_3. Firstly the statements of the American authors that vitamin B_3 is not present in extracts prepared by our methods. Secondly it is most unlikely that vitamin B_3 could stand the alkaline heating, even if it were present in small traces in the marmite. Thirdly we should have to believe that very large increases in the maintenance amount were necessary to convert to rising nutrition. On these grounds, we consider that the factor is not B_3. Hence three factors of the B type must be necessary in pigeon nutrition. It remains to be seen whether the unknown is identical with the rat factors B_2 or B_4.

Vitamin B_2. All workers have not the same criterion for this vitamin. Goldberger *et al.* [1926] and Chick and Roscoe [1929] employed an autoclaved yeast as source of this factor. Hassan and Drummond [1927], and Reader [1930] used an alkalised yeast extract (marmite). Both Chick and Roscoe, and Reader however have agreed that the Y concentrates are free from vitamin B_2. YB was recently tested in this laboratory for vitamin B_2, which was found to be completely absent (Reader, personal communication). The unknown factor cannot therefore be vitamin B_2.

Vitamin B_4. At first we suggested that the third pigeon supplement might be identical with the recently characterised vitamin B_4 [Reader, 1930], though we were careful to point out that this was not proven. Colour was given to this suggestion by the knowledge that the Y preparations contained vitamin B_4. Further investigation has justified the caution. Two classes of evidence may be produced against the identity of the unknown with vitamin B_4.

X preparations which contained no or little vitamin B_4 do not give maintenance when supplemented with vitamin B_4. Other evidence against it is obtained by the use of Reader's alkalised marmite [Reader, 1930]. This is free from vitamins B_1 and B_4, and as we have stated previously does not behave as a supplement to polished rice (Harben lectures). A falling nutrition upon vitamin B_1 (given as an X preparation) can be converted into maintenance by giving in addition alkalised marmite, 1·5 g. *per diem*. The results obtained depend in part upon the weight. The typical chart given in Fig. 4 is for two

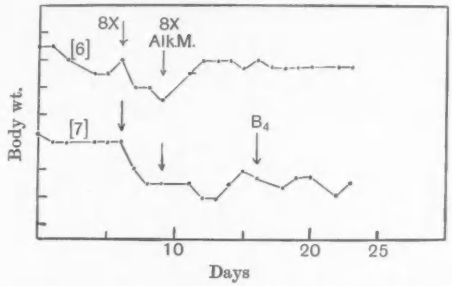

Fig. 4. B_1 and alkalised marmite. Ordinates. Each division 10 g. Body weight on 20th day; bird 6, 317 g.; bird 7, 427 g. 8X means 8 doses daily of vitamin B_1 as X preparation

birds (Nos. 6 and 7); the fall upon rice was not appreciably changed by giving 8 B_1 as an X preparation. In our experience it would have continued; it was checked by giving additions of alkalised marmite (1·5 g. daily) to the vitamin B_1. In other experiments the combination of X and alkalised marmite has produced maintenance for still longer periods.

The particular instances given in Fig. 4 were produced by an X preparation subsequently found to contain vitamin B_4. The weight drop upon vitamins B_1 and B_4 alone is additional evidence that B_4 is not the unknown factor. Further addition of vitamin B_4 in bird 7 did not give rising nutrition, which supports the view that the factors B_3 and B_4 are not identical. From the above it is concluded that vitamin B_4 is not the missing entity.

We may summarise by saying that the supplementary factor present in Y preparations, necessary together with vitamin B_1 and B_3 for maintenance nutrition in the pigeon, is not vitamin B_4, as was suggested earlier, or vitamin B_2. It is stable to an alkaline heating which will destroy vitamins B_1 and B_4. Hence it appears to be a second alkali-stable factor, which can be regarded provisionally as vitamin B_5, recognising that this term is used purely for convenience until further work is produced. Direct proof must await the concentration of the factor concerned.

The accompanying table will clarify our conclusions as to the content of factors in the different experimental preparations, + = present, − = absent, trace = present in small amounts, ? = unknown. The data as to vitamins B_2 and B_4 are taken from the papers of Reader.

Substance	B_1	B_2*	B_3	B_4*	[B_5]
Whole wheat	+	+	+	?	+
Steamed wheat	−	?	+	?	+
Fresh marmite	+	+	Trace	+	+
Alkalised marmite	−	+	−	−	+†
50 % alcohol extracts of norite (Y)	+	−	Trace	+	+
N/10 HCl extracts of norite (X)	+	−	[Trace?]	−‡	−

* Vitamins B_2 and B_4 have not been shown to be needed by the pigeon.

† A few experiments indicate that vitamin B_5 in YB is not destroyed by heating for 35 mins. at 100° in presence of *N*/2 NaOH. Such a treatment we have found previously destroys vitamin B_1.

‡ X preparations have been sometimes found to contain vitamin B_4 (Reader).

Discussion.

Of the four recognised B factors, B_1 has been shown to be needed by the pigeon and the rat, B_2 and B_4 only by the rat, and B_3 only by the bird. Hence not more than three factors have been claimed as necessary for any given animal.

The essential point established in this paper is that three B factors are necessary for pigeons, as we have previously suggested (Harben lectures). We have taken the view that vitamins B_3 and B_4 are different, and shown that the unknown is not identical with either. It must be realised that the recognition of three pigeon factors as necessary carries with it the implication that either vitamin B_1 or B_5 could under certain circumstances behave like vitamin B_3, *i.e.* induce rise to maximum weight. These would be conditions in which the other two were previously present, so that the third was the limiting factor. We think that this has complicated some of our experiments, especially now that we have learnt that vitamin B_3 is stable to heat under certain conditions.

All X preparations do not appear to be equally free from vitamin B_5, and therefore do not all show the same marked difference from Y. The X preparation described in Part I was remarkably free from the other factors. As the method of preparation is still largely empirical, this difference between different preparations is to be expected. Our most recent work (above) has convinced us that an alkaline treatment of marmite which destroys vitamins B_1 and B_4 will leave sufficient vitamin B_5 to supplement small doses of vitamin B_1 given as X. This is apparently contradictory to our previous curve (Harben lectures), showing a case in which three doses of vitamin B_1 plus alkalised marmite did not give maintenance. The experiment was performed before the conditions of autoclaving were well defined, and before it was realised that 1·0 g. of alkalised marmite did not supply enough of the alkali-stable factor. Both these effects were probably operating to produce the different result.

We have not referred either to the heart block effect [Carter and Drury, 1929] or to the work of Rosedale [1929]. It does not yet seem clear that the heart block factor is to be classed in the B group. The effects obtained by Rosedale upon intestinal stasis are produced by a factor precipitated from rice polishing extract by neutral lead acetate. Since the factor with which we have been dealing is present in the lead acetate filtrates from yeast, it would appear to be different from that of Rosedale; we would not care however

to be dogmatic upon such a point owing to the well-known differences in the behaviour of the same factor in different materials.

Experimental methods. These have been essentially the same as in Part I, and therefore need no separate description.

Summary.

These conclusions apply to pigeons which have been nourished upon complete diets within a period of 35 days of commencing the rice feeding.

1. Three B factors are necessary for nutrition in the pigeon.

2. The factor which supplements vitamin B_1 in the production of maintenance nutrition is present in 50 % alcohol extracts of the charcoal (Kinnersley and Peters) process for concentrating vitamin B_1, and also in alkalised marmite. It is not identical with vitamin B_3, or B_2 or B_4 and can be termed vitamin B_5.

3. Whole wheat steamed for 5 hours gives rising nutrition when supplemented with small doses of vitamin B_1. Vitamin B_3 (Williams and Waterman) is therefore heat-stable under some conditions.

4. Pigeons given enormous doses of vitamin B_1, up to 36 doses *per diem*, behave normally.

5. Storage of vitamin B_3 may influence markedly the behaviour of birds up to some 20 days after being placed upon polished rice.

We are grateful to Dr Reader for kindly supplying the preparations of vitamin B_4 and of alkalised marmite used in this research, and for performing several tests for vitamin B_4 upon our extracts. We wish also to thank W. Wakelin for skilful assistance, willingly rendered. Our thanks are due to the Medical Research Council for a personal grant to one of us (H. W. K.) and for a grant for expenses. We are also grateful to the Government Grant Committee of the Royal Society.

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CCX. THE EFFECT OF HYDROGEN ION CONCENTRATION UPON THE PRECIPITATION OF CERTAIN BASIC SUBSTANCES BY PHOSPHOTUNGSTIC ACID.

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(*Received November 1st, 1930.*)

THOUGH it has been known in a general way that acidity affects the precipitation of basic substances by phosphotungstic acid, it is not usually realised that the chemical constitution of the base has a marked influence upon the p_H at which precipitation occurs. The observations recorded below have been applied already in the concentration of vitamin B_1 [Kinnersley and Peters, 1930], and experiments are in progress to ascertain how far the method can be generalised[1].

EXPERIMENTAL.

Phosphotungstic acid (Mercks' puriss.) 10 % is carefully brought with 20 % NaOH to approximately p_H 6·0. The reagent should not be made actually alkaline during this process. The substance for test in a concentration of at least 1·0 mg. per cc., is brought to p_H 7–8, and 1–2 drops (1/20 cc.) of the phosphotungstate are added. If there is no opalescence, $N/100$–$N/10$ H_2SO_4 is cautiously added from a micro-burette until a faint opalescence appears. Such opalescence is an indication of commencing precipitation, and upon standing for some hours precipitation at the indicated p_H becomes complete. No precipitation appears to take place upon standing unless the opalescent stage has first been reached.

The p_H at which the precipitate first appears differs markedly for different bases. For instance, whereas histamine first precipitates at p_H 7·4–7·8, ammonium salts do not precipitate until the solution has a p_H of 1–2. Bases therefore which precipitate towards the more alkaline reactions can be freed from ammonium salts by this treatment. The only exception to the rule that these precipitates tend to dissolve in alkaline solution and to reappear in acid is guanidine, with which the precipitation over the range p_H 3–10 takes place in the opposite sense.

Table I shows the results obtained by this method for several bases.

The estimations of p_H were made by a colorimetric method and cannot be relied upon to more than $\pm 0{\cdot}15$ p_H.

[1] A preliminary account appeared in *Chem. and Ind.* (1930) *Proc. Biochem. Soc.* p. 517.

Table I. p_H *and precipitation of bases.*

Compound*	Conc. mg. per 1·00 cc.	p_H
1. Histamine	5·0	7·8
2. Aminomethylglyoxaline	3·5	6·8
3. 4 (or 5)-Glyoxaline methyl ethyl carbinol	3·3	4·8†
4. Glyoxaline-4 (or 5)-formaldehyde	3·9	3·8
5. 4 (or 5)-Methylglyoxaline	3·7	3·8
6. Arginine (nitrate)	2·3	3·7
7. 4-Hydroxy-2–6-dimethylpyrimidine	5·2	3·6
8. Lysine (picrate)	5·1	3·3
9. *dl*-α-Hydroxy-β-glyoxaline-4 (or 5)-propionic acid	3·8	3·2
10. 2-Hydroxy-4–6-dimethylpyrimidine	5·2	3·2
11. Glyoxaline-4 (or 5)-acetic acid	4·1	3·0
12. Choline	4·6	2·9
13. 2-Methylglyoxaline	2·6	2·9
14. Creatinine	5·0	2·9
15. Histidine	5·0	2·8
16. Ergothioneine	—	2·5
17. 2-Thiol-4 (or 5)-amino-methylglyoxaline	4·7	2·2

The p_H recorded is that for the appearance of the first opalescence.

* Compounds 3, 7, 10, 16, 17 were kindly supplied by Dr J. M. Gulland, compounds 5, 13 by Prof. Pyman, and 9 and 11 by Prof. Barger. The remaining compounds were from commercial sources.

† p_H somewhat doubtful. A sample of the platinum salt kindly supplied by Dr Gulland precipitated at p_H 2·8 in conc. 4 mg./cc.

Certain tentative observations can be made. The unsubstituted glyoxaline compounds appear to precipitate at about p_H 3·0, and this point is not much affected by combination with acetic or hydroxypropionic acid. The aldehyde group appears to change the point of precipitation to the alkaline side, as does a long side chain. The base histamine with a 2-carbon chain attached begins to precipitate actually at an alkaline p_H, and aminomethylglyoxaline at a slightly less alkaline reaction. The introduction of —SH seems to alter the precipitation point markedly to the acid side of p_H 3·0, which is the point at which the simple glyoxaline compounds precipitate.

Guanidine compounds really show two points of precipitation, one alkaline and one acid, which vary according to the degree of substitution of the compound. If a guanidine solution of p_H 5·0 approx. is treated with phospho-

Table II. *Guanidine compounds.*

Substance	Conc. mg. per cc.	p_H	Notes
Guanidine sulphate	5·0	7·0	Precipitate not redissolved by alkali; redissolves upon increasing acidity to p_H 4·0, but is reprecipitated in 5 % sulphuric acid
Aminoguanidine	15	8·5	Redissolved by excess alkali; precipitated by strong sulphuric acid
asym-Dimethylguanidine	5	Nil	Not precipitated at all by alkali, but precipitated in 5 % sulphuric acid
Methylguanidine	5	8·5	Faint precipitate, redissolved by alkali, precipitated by 5 % H_2SO_4
Arginine nitrate	5	7·0	Redissolved by alkali, further precipitate appears on the acid side at p_H 3·7

The p_H given in this table refers to the point at which a precipitate first appears upon passing from a more acid to a less acid reaction.

tungstic acid, no precipitate appears. Addition of alkali to p_H 7·0 induces precipitation, as also does acidification to p_H 1·0. Table II embodies the observations made.

It seems to be clear that for the alkaline precipitation the NH_2 group must be intact. Introduction of methyl or NH_2 reduces the tendency to precipitate, and dimethyl suppresses it. Arginine shows a faint tendency to precipitate. It is to be noted that the two precipitation reactions for arginine appear to be quite distinct.

The relation between the concentration of the base and the p_H at which precipitates first appear has been studied more fully for creatinine, guanidine, and less fully in the case of histidine and histamine. The curves in Fig. 1

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for o Creatinine
× Histamine

read o Creatinine
× Histidine

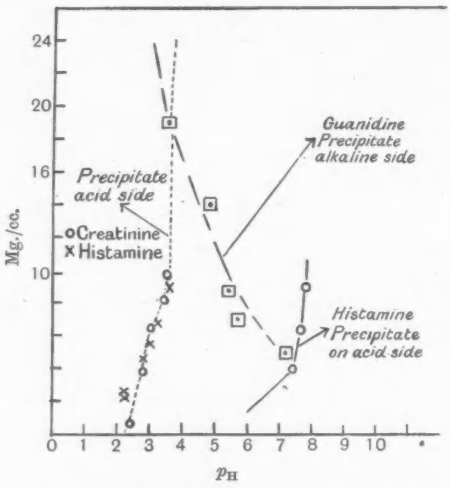

Fig. 1. p_H and phosphotungstate precipitation.

show the results obtained. For histamine, below 1 mg. per cc. most of the compounds become too soluble to obtain results. For histidine and creatinine a variation of concentration from 1 to 10 mg. per cc. changes the p_H for precipitation from 2·5 to 3·5 approx. With creatinine the curve rises somewhat more steeply. A variation in concentration of six times only affects the p_H of precipitation by some 0·8 p_H. For neutralised guanidine sulphate it will be seen that the curve for alkaline precipitation rises much less steeply.

It would be unwise to draw conclusions as to the nature of these precipitates until more work has been done, but since they do not tend to appear until some of the compounds are neutralised, it would seem that the bases are precipitating as salt complexes with the phosphotungstates. Some evidence has been obtained that in mixtures of bases there is a shift in the positions of these precipitation points. It is likely that these results explain certain

difficulties which have appeared in the decomposition of phosphotungstic acid precipitates. It would clearly be very difficult to decompose a guanidine phosphotungstate of this nature by baryta. In most cases re-solution of the phosphotungstates mentioned in Table I is to be obtained by taking to a point approximately 2·0 p_H units more alkaline than that of precipitation.

SUMMARY.

The hydrogen ion concentration at which phosphotungstate precipitates of certain bases appear depends to a large extent upon the chemical constitution of the base. With most of the bases studied the precipitation appears upon passing from a more alkaline to a less alkaline solution; with guanidine compounds, this is reversed.

REFERENCE.

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CCXI. THE RELATION OF HYDROGEN ION CONCENTRATION TO THE PRECIPITATION OF PURIFIED TORULIN (YEAST VITAMIN B_1) BY PHOSPHOTUNGSTIC ACID.

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(*Received November 1st, 1930.*)

In a previous communication [Kinnersley and Peters, 1927] we described methods by which torulin (yeast vitamin B_1) could be concentrated to an activity in our terms of 0·025 mg. *per diem* (pigeon dose). The object of this paper[1] is to describe certain improvements in obtaining material of this activity, especially the use of new methods of phosphotungstic acid fractionation. At the same time, we propose to mention some of our experiences with other methods of fractionation. It must be stated at once that we have not yet reached an activity greater than 0·01 mg. *per diem* as the average dose for several pigeons, but we believe that the methods described here are in some respects simpler than others, and may be capable of application in other fields. A specimen of the crystals of the Jansen and Donath vitamin [1926] from rice polishings which we have recently had the opportunity of testing [Jansen, Kinnersley, Peters and Reader, 1930] was 40 % more active than the preparations described here.

Experimental.

The method previously described by us involved adsorption upon charcoal by a technique which is now standardised, subsequent treatment of the charcoal extracts with sulphuretted hydrogen to remove metals, and fractionation with alcohol with gradual change in p_H of the extract. After this the active material was obtained by the use of phosphotungstic acid and platinic chloride, following the Jansen and Donath method in the main, with the exception that the decomposition of the phosphotungstate was carried out by the classical method of grinding with solid baryta. The behaviour of these yeast extracts to phosphotungstic acid is a very curious one. At an earlier stage in our procedures, before the baryta treatment had been introduced, we found that it was impossible to recover the vitamin from the phosphotungstates, owing we now believe to its precipitation with traces of gum. Later in the preparation, after removal of these impurities, the active substance can be

[1] A preliminary account has appeared, *Chem. Ind.* (1930) *Proc. Biochem. Soc.* p. 517.

recovered from a phosphotungstic acid precipitate; but we have never been able to recover it by the use of acetone and baryta, as described by Jansen and Donath for the rice vitamin, and also confirmed by us [Peters, 1930, 1]. We do not understand why this is so, but it forms an admirable illustration of the manner in which the apparent properties of these more active biological substances may change during the course of a purification. It is safe to say that at each stage of purification it is wise to treat the material present as a fresh chemical problem and not to assume that a process rejected at an earlier stage may not be applied with success at a later. We give below an experiment illustrating that the baryta-acetone method cannot be applied to the yeast vitamin, even when this has reached the purity of 0·8 mg. *per diem*.

Exp. 1. *Decomposition of phosphotungstate by baryta-acetone.*

A phosphotungstate precipitate of torulin was made by adding to a torulin solution (475 cc.) 30 cc. of 10 % phosphotungstic acid neutralised to p_H 6·0 together with sulphuric acid to p_H 4·5. The precipitate was extracted twice with 50 % acetone, and the extract centrifuged.

Centrifugate

25 cc. of cold saturated baryta solution added until alkaline to phenolphthalein, and the whole thoroughly stirred.

Centrifugate

Sulphuric acid added to p_H 1·0, and $BaCl_2$ to remove excess of H_2SO_4.
Solids 468 mg.
Activity nil.

Insoluble

The two insoluble portions were extracted by grinding with solid baryta and a little water several times.

Centrifugate

Ba removed, acidified, alcohol added to 20 % and kept in ice chest.
Organic solids 285 mg.
Doses present 6000.

Insoluble (discarded)

Insoluble

The activity in the final centrifugate corresponded very closely to the number of doses originally present, showing that none of the activity had passed into solution by decomposition with baryta-acetone. It will be recalled that the Dutch workers employ baryta-acetone for the decomposition of their phosphotungstates from rice polishings. It is curious that the method should not be applicable in the purification of torulin.

Among the attempts made by us to eliminate the use of metals in the later stages of our fractionation was an extensive use of mixed solvents, especially ether, alcohol and acetone. It does not seem to be worth while to give in detail the many experiments made: one of them has been presented elsewhere [Peters, 1930, 1]. A marked feature of the experiments, which were carried out with material of activity of 0·08 mg., was the disappearance of activity whenever the conditions were those of dry ether-alcohol. It was found that this disappearance of activity was especially marked when the initial p_H of the substance for fractionation was less acid than p_H 4·5. Quite small traces of water influenced the result as to whether the substance was thrown out by

alcohol-ether or not. For maximal removal of inactive substances, conditions must be kept at as alkaline a point and as anhydrous as possible. Unfortunately these conditions are just those which are found to lead to instability. We have excluded from our experiments the possible inactivating effect of impurities in the solvents by careful purification, and are therefore satisfied that the effects are not due to adventitious impurities. It is quite remarkable to find how unstable torulin is to alkali in alcoholic solution. We have several experiments upon this point, but think that only one is worth quoting. Exp. 2 shows the loss of activity when changing the p_H in alcohol by the addition of aqueous NaOH.

Exp. 2. *Effect of NaOH in the cold on alcoholic solution of torulin.*

The torulin solution used contained 4·060 g. of organic solids and 10150 doses.

The 98 % alcoholic solution was treated at room temperature with 2·5 cc. of 20 % NaOH in 5 cc. of alcohol, making the final p_H about 5·2. The precipitate so formed was removed upon the centrifuge.

Centrifugate	Precipitate
Doses 3000 (2)[1].	

The precipitate and filtrates were then combined and concentrated, subsequent tests giving 3700 doses present (2).

It must be concluded that though small amounts of impurities can be removed from material at p_H values on the acid side of 4·0 by the use of mixed solvents, the method is not reliable.

Adsorption by barium phosphotungstate.

The occasional losses experienced especially in the less pure concentrate led us to try whether the base was adsorbed by barium phosphotungstate *per se*. The following experiment shows that it is not.

Exp. 3. 80 doses of torulin of activity 0·12 mg. in solution in 3·0 cc. of water were treated with a precipitate of barium phosphotungstate made by adding to 1·4 cc. of 10 % phosphotungstic acid 2–3 cc. of hot baryta together with some solid baryta: the precipitate was centrifuged and ground with more solid baryta. The combined filtrates were brought to p_H 1·0 with sulphuric acid.

The tests showed 80 (3) doses meaning that the activity was still present, and therefore that any losses of torulin at a phosphotungstic stage are not likely to be due to adsorption by the barium phosphotungstate.

It will be observed that no regeneration of activity occurred in Exp. 2 upon adding together the two fractions. We have often tried to obtain this effect upon a sudden loss of activity but have never observed it, and cannot therefore confirm the experience of Guha and Drummond [1929].

Improvements in the use of phosphotungstic acid.

In the attempt to reduce the laborious operations involved in working up the charcoal concentrates by our original technique, we noticed that the

[1] Average of tests given throughout, the number of tests upon which it is based being added in brackets.

vitamin was very largely precipitated even when a marked defect of phosphotungstic acid was present. This led us to develop some observations made previously upon the effect of p_H upon the precipitation of bases by phosphotungstic acid [Peters, 1930, 2]. In brief, it is found that if neutralised phosphotungstic acid (p_H 6·0) is added to solutions of bases initially at p_H 7·0 followed by sulphuric acid, precipitation of certain bases seems to take place rather sharply at a p_H dependent upon the nature of the base. In the case of the vitamin, we brought the material for fractionation to p_H 7·0 approximately, added 10 % phosphotungstate (p_H 6·0) and sulphuric acid alternately drop by drop until commencing precipitation as indicated by opalescence appeared. After standing, the various fractions were removed at various hydrogen ion concentrations. Some substances present precipitate sharply at p_H 7·0: others as the p_H is changed to 5·0. Exp. 4 illustrates in detail one of the earlier experiments bearing upon this, and shows the rough grouping of the precipitates between p_H 7·0 and 1·0 approximately.

Exp. 4. *Fractional precipitation at different* p_H *values.*

The torulin used for the experiment was obtained from the decomposed phosphotungstate obtained from a charcoal concentrate fractionated first with alcohol and later with phosphotungstic acid at an acid p_H.

Organic solids 36 mg., containing approximately 170 doses.

The alcohol was removed after addition of a little water. At a volume of 6·0 cc. previously brought to p_H 8·0, the neutralised phosphotungstate was added until no further precipitate appeared. A series of precipitates was obtained, keeping the phosphotungstic acid in slight excess at each stage, and gradually increasing the hydrogen ion concentration by the addition of sulphuric acid. The precipitates so obtained were decomposed by grinding with solid baryta several times in a small centrifuge tube and removing the barium phosphotungstates etc. by centrifuge and filtration. The excess Ba was removed with addition of sulphuric acid, and each acidified to Congo red with HCl where necessary. 10 % alcohol was added for preservation.

	Precipitates p_H	Nature	Corresponds to fraction	Organic solids mg.	Bird test
1	8·0–8·5	Bulky yellow	Guanidine	5·2	Less than 20 doses
2	8·0–5·5	Small „	Histamine	3·3	18
3	5·5–4·6	„ „	—	2·8	37
4	4·6–3·5	„ „	—	?	130
5	3·5–2·0	Large white	Choline	15·6	Nil
6	More acid than 2·0	„ „	Ammonium salts, etc.	—	?
			Total	26·9	

Though the above tests were not made upon many birds, they leave no doubt in the mind that the activity lies between p_H 8·0 and 3·5 under these conditions. It will be seen that the total amount of solids recovered is satisfactory. The details of column 4 are given to indicate to some extent the substances which might be expected to come down at these p_H values.

It will be noted that the precipitation of the vitamin appears to start at p_H 7·0–7·5.

Further experiments upon these lines and upon a rather small scale showed that if the precipitate which came down between p_H 7·0–5·0 was gradually defined and precipitated upon successive occasions in smaller volume, it was possible to attain a high degree of concentration by using these methods. It

was however found best to insert an alcohol precipitation stage between successive fractionations. In one case by this method, we obtained in two stages from torulin of activity 0·1 mg. a small amount of torulin of activity 0·012 mg. We give below the details of a larger scale experiment, which illustrates as clearly as possible the best method known to us of carrying out this fractionation.

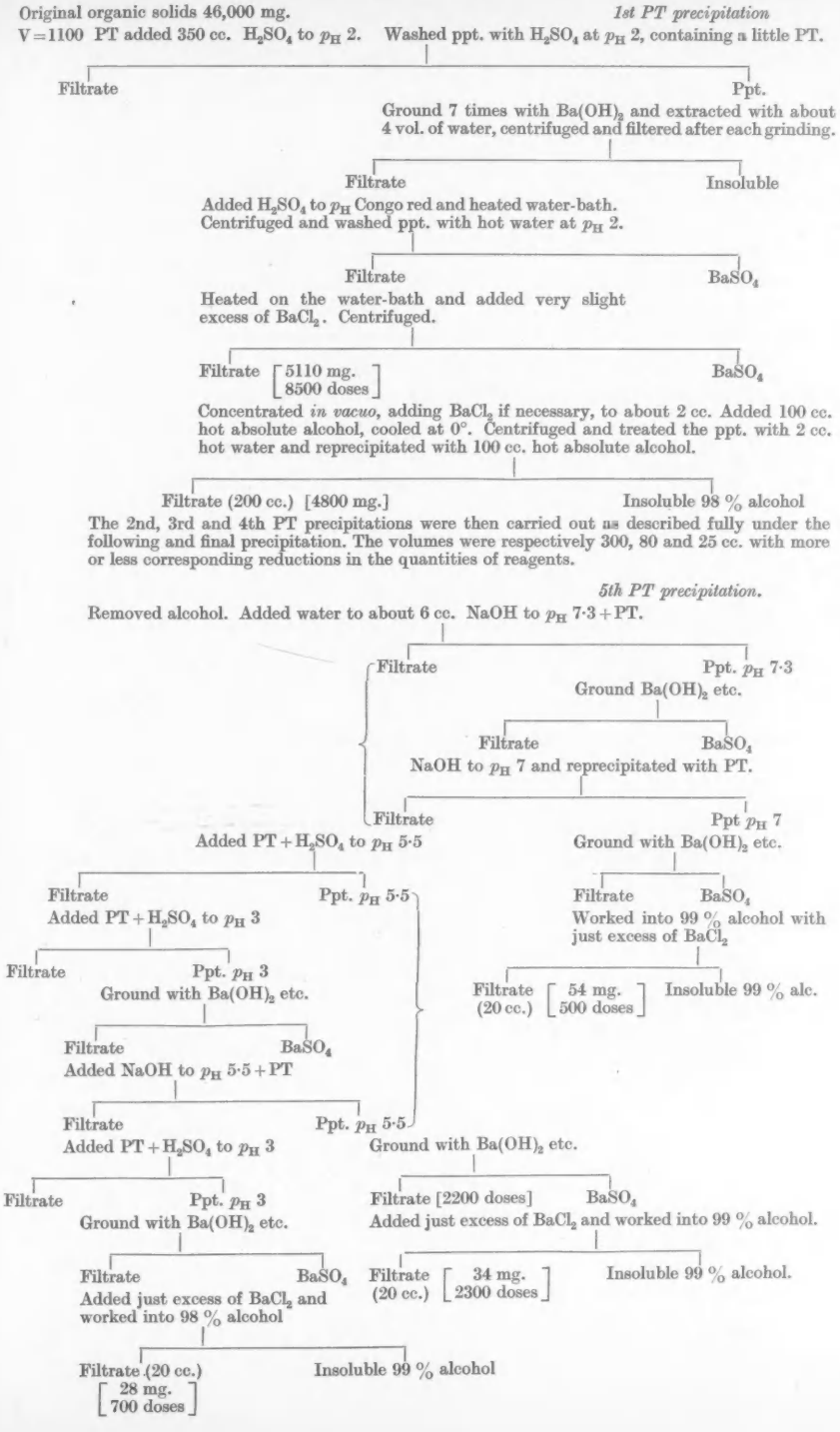
Without giving the experiments in detail, we may say that we have not found the methods applicable to torulin before the charcoal adsorption stage. Immediately after this stage however fractionation can be applied with a considerable degree of success, and we have been able to eliminate the metals present without H_2S treatment. This is however somewhat uncertain so that we believe that it is advisable to remove metals by our customary technique.

Immediately after this removal and elimination of the H_2S fractional precipitation can be applied. The limits of the precipitation are less sharp in the early stages, so that it is necessary to collect the precipitate over the range p_H 5·0–2·0 in order to include the greater bulk of the vitamin. This precipitate is usually known as the first phosphotungstic precipitate.

The material is worked up by repeated extraction with solid baryta and minimal amounts of water, the process being repeated five times and in some cases more.

After grinding in the centrifuge tube with the moistened baryta, the precipitate is centrifuged, and the cloudy supernatant fluid filtered through a small paper into another centrifuge tube. This is necessary to remove the last traces of phosphotungstate. The combined filtrates are treated with sulphuric acid to remove excess of baryta, and with HCl or H_2SO_4 until acid to Congo red. The precipitate is centrifuged, and extracted with warm acid (approx. $N/100$). If this is not done, some of the bases may adhere to the precipitate. A second or further phosphotungstate precipitation is carried out by bringing the solution to p_H approx. 7·0, and then adding as before alternately sulphuric acid and phosphotungstate, separating off the precipitates as they appear. It will be found that substances can be defined to some extent by the separation of groups of precipitate. If the precipitate is colloidal, sodium sulphate can be added to ensure complete precipitation. It is also well to cool in ice water. A faint opalescence will usually be found to yield a good precipitate when the solution is allowed to remain for 2 hours at 0°. Each subsequent step of phosphotungstate precipitation is carried out in a smaller volume than the one preceding. Before describing this experiment, we wish to draw attention to a point which may influence results obtained with the pigeon tests. As was stated in one of our earlier papers, it has been our custom to work with slight excess of barium chloride in our solutions owing to the destructive effect of traces of sulphuric acid at any stage approaching dryness. Extraction of dried vitamin by absolute alcohol removes mere traces of such barium chloride together with the vitamin; but these traces have been sufficient to interfere with the test and in some cases to inhibit the effect altogether. We have not

Table I. *Exp.* 6.


Original organic solids 46,000 mg.
V = 1100 PT added 350 cc. H_2SO_4 to p_H 2.
1st PT precipitation
Washed ppt. with H_2SO_4 at p_H 2, containing a little PT.
Filtrate
Ppt.
Ground 7 times with $Ba(OH)_2$ and extracted with about 4 vol. of water, centrifuged and filtered after each grinding.
Filtrate
Insoluble
Added H_2SO_4 to p_H Congo red and heated water-bath. Centrifuged and washed ppt. with hot water at p_H 2.
Filtrate
$BaSO_4$
Heated on the water-bath and added very slight excess of $BaCl_2$. Centrifuged.
Filtrate [5110 mg. 8500 doses]
$BaSO_4$
Concentrated *in vacuo*, adding $BaCl_2$ if necessary, to about 2 cc. Added 100 cc. hot absolute alcohol, cooled at 0°. Centrifuged and treated the ppt. with 2 cc. hot water and reprecipitated with 100 cc. hot absolute alcohol.
Filtrate (200 cc.) [4800 mg.]
Insoluble 98 % alcohol
The 2nd, 3rd and 4th PT precipitations were then carried out as described fully under the following and final precipitation. The volumes were respectively 300, 80 and 25 cc. with more or less corresponding reductions in the quantities of reagents.
5th PT precipitation.
Removed alcohol. Added water to about 6 cc. NaOH to p_H 7·3 + PT.
Filtrate
Ppt. p_H 7·3
Ground $Ba(OH)_2$ etc.
Filtrate
$BaSO_4$
NaOH to p_H 7 and reprecipitated with PT.
Filtrate
Ppt p_H 7
Added PT + H_2SO_4 to p_H 5·5
Ground with $Ba(OH)_2$ etc.
Filtrate
Ppt. p_H 5·5
Filtrate
$BaSO_4$
Added PT + H_2SO_4 to p_H 3
Worked into 99 % alcohol with just excess of $BaCl_2$
Filtrate
Ppt. p_H 3
Filtrate (20 cc.) [54 mg. 500 doses]
Insoluble 99 % alc.
Ground with $Ba(OH)_2$ etc.
Filtrate
$BaSO_4$
Added NaOH to p_H 5·5 + PT
Filtrate
Ppt. p_H 5·5
Added PT + H_2SO_4 to p_H 3
Ground with $Ba(OH)_2$ etc.
Filtrate
Ppt. p_H 3
Filtrate [2200 doses]
$BaSO_4$
Ground with $Ba(OH)_2$ etc.
Added just excess of $BaCl_2$ and worked into 99 % alcohol.
Filtrate
$BaSO_4$
Filtrate (20 cc.) [34 mg. 2300 doses]
Insoluble 99 % alcohol.
Added just excess of $BaCl_2$ and worked into 98 % alcohol
Filtrate (20 cc.) [28 mg. 700 doses]
Insoluble 99 % alcohol

had the opportunity to investigate this matter. It would seem to be some chemical effect, as the amount of barium present would not be enough to be toxic: in practice we add sulphuric acid to each test (one drop of $N/10$ H_2SO_4) to ensure the absence of barium. A few experiments have indicated that the vitamin is soluble in absolute alcohol even in the presence of barium chloride both at p_H 2·0 and 5·0, but that it is no longer soluble at p_H 5·0 in strong alcohol if it is converted to the sulphate.

We record the full details of an experiment in which a sample of vitamin was carried through 5 phosphotungstate precipitations without much loss.

Exp. 5. The starting material was a 50 % acid alcoholic extract of vitamin adsorbed upon norite charcoal, worked up commercially to this stage by Messrs Burroughs Wellcome and Co. (to whom our best thanks are due). Organic solids present 45·7 g. The alcohol was removed upon the water-bath, and metals by treatment with H_2S at p_H 3·5–4·0 as usual. After concentration at p_H 3·0, water was added to a volume of 1100 cc., followed by 350 cc. of neutralised phosphotungstic acid (10 % Merck at p_H 6·0) then sulphuric acid to p_H 2·0. The precipitate was centrifuged and washed once. The subsequent treatment is given in Table I. All phosphotungstate precipitates unless otherwise stated were ground with solid baryta, and extracted with a little water (about 3–4 times the volume of the precipitate), centrifuged and the supernatant fluid filtered. This process was repeated with each precipitate some five times. The combined filtrates were treated with H_2SO_4 to remove the greater part of the barium, and with HCl until acid to Congo red, leaving a very slight excess of $BaCl_2$. The precipitate was re-extracted with approx. $N/100$ acid. In the following description, Vol = volume, PT = phosphotungstate solution of p_H 6·0, and AA = absolute alcohol.

Table II summarises the experiment. Volume refers to volume at the stage of phosphotungstate precipitation.

Table II.

Stage		Volume cc.	Org. solids g.	Total doses	Activity mg.
1st phosphot. ppt.	p_H 7·0–2·0	1100	5·110	? 8000	? 0·6 probably 1·0
2nd phosphot. ppt.	p_H 6·0–5·0	300	0·653	3500	0·5
3rd phosphot. ppt.	p_H 7·0–5·0	80	0·265	4200	0·06
5th phosphot. ppt.	p_H 7·0–5·5	25	0·050	1760	0·035
Final ppts.*	p_H to 7·4	—	0·054	500	0·1
	p_H 7·4–5·5	—	0·034	2300	0·012
	p_H 5·5–3·0	—	0·028	700	0·04
			Total	3500	

* Collected precipitates.

It will be noticed that, considering all the operations and the degree of concentration achieved, the recovery in the last stages is good. It is probably much better than this all through the experiment because from another experiment carried out on the same material it appears that the first two birds must have given abnormally high tests. There would seem to be little doubt

that the important fraction is that precipitating between the limits of p_H 7·4–5·5 and that this will prove to be a property of pure torulin. By the use of phosphotungstic acid alone, the activity has been increased from 1·0 mg. approx. per day to 0·012 mg., or some 100 times. The final material is mainly crystalline and has a little more than half the activity of the rice vitamin crystals of the Dutch workers. The experiment lends additional support to the view that we are dealing with a basic substance.

We have tried to apply this method to the direct $N/10$ HCl extracts of norite charcoal in preparations starting initially from baker's yeast, but find that it does not succeed unless there is a preliminary stage of concentration and removal of metals by H_2S. The concentration can readily be effected upon the open water-bath, at p_H 2·0 approximately, without damage to the activity. After the collection of the first phosphotungstic precipitate, the second treatment with phosphotungstate does not succeed well unless an alcohol stage is inserted, as in Exp. 5. Preparations worked up from a British Drug Houses concentrate have however been treated successfully with phosphotungstate direct, without preliminary removal of metals. We believe however that the H_2S stage is worth the time which it occupies[1]. When applied to a $N/10$ HCl extract of the norite charcoal of initial activity approximately 0·5 mg., one may expect to reach 0·3 mg. or under at the first phosphotungstate stage, and 0·06–0·07 mg. in the precipitate at p_H 7·0–5·0 at the second stage. It is to be noted that the amounts of phosphotungstic acid required for this method of fractionation are comparatively small, thereby effecting economy in the use of a rather expensive reagent.

Summary.

A method is described of preparing a torulin (yeast vitamin B_1) concentrate of activity 0·012 mg. by the use of fractional precipitation by phosphotungstic acid at varying p_H values.

Certain further experiments upon torulin are described.

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[1] See accompanying paper for details of the method of handling the British Drug Houses concentrate [Carter, Kinnersley and Peters, 1930].

CCXII. THE ANALYSIS OF THE OSMOTIC PRESSURES OF THE SERUM PROTEINS, AND THE MOLECULAR WEIGHTS OF ALBUMINS AND GLOBULINS.

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I. *Introduction.*

The determinations of the molecular weights of serum albumin and globulin made by Sørensen [1925], Adair [1926] and Svedberg and Sjögren [1928] show a considerable range of variation which Svedberg has attributed to the instability of these proteins. In the work recorded in this paper, methods for eliminating errors due to instability of proteins have been investigated, and comparisons have been made of molecular weights of serum albumin and globulin from different sources, purified by different methods. The values of the molecular weights given by Sørensen and by Adair were obtained by osmometric methods, which have also been used in this work.

If the protein be equilibrated with a salt solution at the physiological hydrogen ion concentration, a part of the observed osmotic pressure, symbolised p_i, may be attributed to the unequal distribution of the diffusible ions. In ideal solutions p_i is determined by the formulae of Donnan [1911]. It has been suggested that these formulae can be applied to the serum proteins [Hecht, 1925] but the effects of slight deviations from the ideal solution laws are exaggerated in the calculation of p_i, which represents a small difference between two large pressures.

Experiments recorded in this paper show that the protein systems are beyond the range of application of Donnan's formulae. The calculations of p_i given below have been made by a thermodynamical formula derived by Adair [1929, 2]. The partial osmotic pressure of the protein ions is equal to the observed osmotic pressure minus the pressure, p_i, due to diffusible ions.

II. *Methods.*

The methods of preparation of the proteins from horse-serum have been described in detail in a previous paper [Adair and Robinson, 1930]. Serum proteins from three different sources have been examined, and comparisons

have been made between preparations of albumin crystallised once, twice and four times by a modification of the method of Hopkins [1900]. In order to prevent decomposition, the proteins were kept at 0° throughout the preparation, except during the crystallisation of albumin, and during the process of centrifuging.

The electrolyte content of the protein solutions was fixed by dialysis in collodion membranes against standard phosphate buffers, a method that is less likely to cause changes in the protein than the alternative procedure in which the protein is first dialysed against water until free from electrolytes and then mixed with the salts required in the final osmometric experiment.

The measurements of osmotic pressure were made with collodion membranes fixed to manometers as described by Adair [1925]. The osmometers were kept in a room at 0° for 15 days or more, the pressures being observed daily. After equilibrium had been established for a number of days, the osmometers were dismantled and determinations of the capillarity correction, the refractive index, the density and the nitrogen content were made on the protein solution. The refractive index and the density of the dialysate were also measured.

The protein concentrations have been stated in terms of g. of dry protein per 100 cc. of solution. In the majority of cases, the protein concentration was calculated from Kjeldahl nitrogen determinations. The factors necessary for this calculation have been redetermined on specially purified material [Adair and Robinson, 1930] and it was found that crystalline horse-serum albumin dried at 110° contained 15·60 % N. The nitrogen content of uncrystallised albumin of horse-serum and that of the albumin of ox- and sheep-serum has not yet been determined; the factor $\frac{100}{15{\cdot}6}$ has been used provisionally for the calculations of the concentrations of these proteins. The nitrogen content of total globulin of horse-serum has been redetermined as 15·13 %. This figure has been used in provisional calculations of the concentration of ox- and sheep-serum globulin.

In a few experiments, the protein concentration was determined by the refractometric method, as described in detail in a previous paper [Adair and Robinson, 1930].

In certain cases, the measurements of osmotic pressure were supplemented by determinations of the membrane potential made with a slightly modified form of the apparatus described by Loeb [1922] in which saturated calomel electrodes are dipped into the protein solution and into the dialysate.

The distribution of ions across the membrane was studied in three experiments in which serum albumin was equilibrated with an ammonium phosphate buffer of p_H 7·3. Ammonia was estimated colorimetrically with Nessler's reagent and phosphates were estimated by the method of Bell and Doisy [1920].

III. *The stability of the serum proteins.*

According to the observations of Svedberg and Sjögren [1928], serum proteins are unstable substances, easily decomposed during the process of purification. In this work, three methods for testing the stability of the proteins have been employed: firstly, the degree of constancy of their osmotic pressures has been observed for periods from 12 days to 3 months; secondly, the dialysis fluids surrounding the membranes have been tested for protein derivatives; thirdly, comparisons have been made of the molecular weights of preparations from different sources, and purified by different methods.

The first test was applied to all the osmometric experiments. In the few cases where the condition of constancy of osmotic pressure was not fulfilled, the cause was attributable to imperfections of the membrane. A rapid fall of osmotic pressure indicated that the membrane was permeable by protein, and in such cases protein could be demonstrated in the dialysis fluid. A gradual fall of pressure was caused in a few cases by absorption of the protein by the membrane, as shown by the reduction in concentration of the protein solution. Typical osmometric readings, yielding constant pressures, are recorded in Table I.

Table I. *Stability of albumin and globulin.*

Osmometer readings in cm. of solution at 1·0°.

Exp. HA 20. Horse-serum albumin. Once crystallised		Exp. HA 31. Horse-serum albumin. 4 times crystallised		Exp. G 19. Horse-serum globulin.	
Date 1929	Osmometer reading	Date 1929	Osmometer reading	Date 1929	Osmometer reading
Aug. 9	26·30	Oct. 31	16·40	Nov. 1	10·58
10	19·80	Nov. 1	14·82	2	7·82
11	18·10	2	14·60	3	7·20
12	17·20	3	14·50	4	7·08
13	16·97	4	14·47	5	7·05
14	16·90	5	14·44	6	7·02
15	16·82	7	14·41	7	7·00
	Reset		Reset		Reset
15	20·25	7	12·92	7	4·80
17	17·20	8	14·09	8	6·49
18	17·05	9	14·40	9	6·85
19	17·00	10	14·42	10	6·91
21	16·90	12	14·45	14	7·02
22	16·80	13	14·43	28	7·02
23	16·80	14	14·43		
24	16·80	15	14·43		

In the second test, positive results for the biuret, the xanthoproteic, the ninhydrin and Millon's reactions were obtained only in the few cases in which the osmometer readings indicated that the membranes were permeable by protein. In all the remaining experiments, completely negative results were obtained with these tests, for once crystallised and for four times crystallised albumin, and also for globulin. These negative results in the tests for protein derivatives are of significance, because they indicate that our preparations of four times crystallised albumin did not give rise to the decomposition products

of low molecular weight referred to by Svedberg and Sjögren [1928]. If these derivatives were present, it is probable that they would diffuse across the membranes. If they were not diffusible, their presence would be manifested by discrepancies between the osmotic pressures of the once and the four times crystallised albumin.

The results obtained in the third test, given in detail in Section V, show that the molecular weight of four times crystallised horse-serum albumin is the same as that obtained for once crystallised material. There was furthermore a close agreement between the molecular weight of the albumin prepared from horse-serum obtained from the University Field Laboratories and from Messrs Burroughs and Wellcome. Horse-blood obtained from a knacker gave an albumin with a molecular weight of 67,000 instead of 72,000, but in this case, it was observed that the protein was contaminated with a coloured impurity, even after three recrystallisations.

The results of all three tests show that the stability of these preparations of albumin and globulin appears to be distinctly greater than that of the proteins as prepared by Svedberg and Sjögren. These authors state that in dilute solution serum albumin is decomposed reversibly into particles of low molecular weight. In a truly reversible reaction, which should be governed by the law of mass action, the amount of protein decomposed in unit time should be proportional to the protein concentration. Svedberg's experiments indicate that 29 % of the protein is decomposed in 0·25 % solution, whereas no decomposition takes place in 1·25 % solution. These figures are inconsistent with the law of mass action, and suggest that the decomposition is due to some external factor.

IV. *Methods for the estimation of the molecular weights of serum proteins in non-ideal solutions.*

In calculations of the molecular weight of a protein from measurements of osmotic pressure made with membranes of collodion and similar substances, there are at least three factors which must be considered, the "ion pressure difference," p_i, due to the excess of diffusible ions inside the membrane, the effects of the volume of the protein hydrate and the effects of attractive and repulsive forces between the molecules represented by the osmotic coefficient g_p in formula (1)

$$p - p_i = p_p = RTg_p m_p \qquad \text{.....(1),}$$

p = the observed osmotic pressure, p_i = the ion pressure difference, p_p = the partial osmotic pressure of the protein ions, m_p = g.-mols. of protein per litre of "solvent," calculated by deducting the volume of the protein hydrate from the volume of the solution.

If the molecular weight of the protein is constant, that is, if the degree of polymerisation is unaffected by changes in concentration, and if the effects of the ion pressure difference and of interionic forces are not too great, it is possible to apply the method of extrapolation described below, whereby the

effects of all the three factors are eliminated simultaneously. The conditions under which this method gives valid results have been studied in previous investigations on haemoglobin [Adair, 1924, 1928], and it has been applied to egg-albumin [Marrack and Hewitt, 1929].

It is necessary to work with systems of well-defined hydrogen ion concentration, such as the phosphate buffer mixtures, because the electric charge or valence of the protein ions may vary over a wide range in unbuffered solutions. As a general rule, the concentration of diffusible salts should be greater than 0·05 equivalents per litre of dialysate in order to reduce the ion pressure difference, but in the region of the isoelectric point it is possible to work with more dilute salt solutions.

The most important experimental determinations required are a series of measurements of the osmotic pressures of protein solutions, with a wide range of protein concentration, in equilibrium with a standard solution of electrolytes of well-defined composition. If the relationship between the pressure and the protein concentration approximates towards a straight line, at low protein concentrations, it is probable that the molecular weight of the protein can be calculated by the modification of van't Hoff's law given below:

$$M = 10RT/\pi_0 = 170{,}330/\pi_0 \qquad \text{.....(2),}$$

M = molecular weight in g. of dry protein.

$RT = 760 \times 22{\cdot}412 = 17{,}033$ at 0°.

π_0 = the limiting value of the ratio π in the infinitely dilute solution.

$\pi = p/C$ where p = the observed osmotic pressure in mm. Hg at 0°.

C = g. of dry protein per 100 cc. of solution.

The factor 10 must be included in the formula, as it is customary to express the protein concentration in g./100 cc. rather than in g./litre. The determination of π_0 by extrapolation is discussed below.

The assumptions concerning the ion pressure difference can be tested by the method for the determination of this pressure from membrane potential measurements, described in Section IX. The constancy of the molecular weight of the protein can further be verified by estimation of the ratio π_0 at different concentrations of electrolytes.

Since variations in the concentrations of electrolytes cause large changes in the activity coefficients of the protein ions, as deduced from solubility measurements, it is necessary to verify the assumption that at all concentrations of salts the osmotic coefficient g_p approaches unity as the protein concentration is reduced. In the case of haemoglobin, it has been shown [Adair, 1928] that g_p approximates to unity in solutions at different hydrogen ion concentrations and at salt concentrations ranging from 0·001 to 1·0 g.-mols./litre. This apparent discrepancy between the activity and the osmotic coefficients can be accounted for by the analysis of the activity coefficients [Adair, 1929, 1].

Determinations of π_0 for protein solutions have been made by extrapolation

from a curve showing the relationship between the osmotic pressure and the protein concentration, or by plotting the ratio π as ordinate and C as abscissa [Adair, 1924, 1928]. In the case of the measurements of the osmotic pressure of serum albumin which have been made in this work, it was found advisable

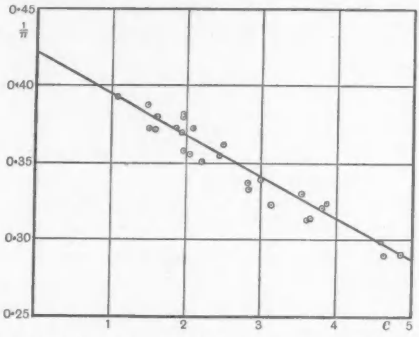

Fig. 1. Crystalline serum albumin at p_H 7·4. Ordinate = pressure-concentration ratio $1/\pi$ (formula 5) at 1°. Abscissa = C = protein concentration in g. per 100 cc. solution.

to adopt a third method, in which $1/\pi$ is plotted as ordinate and C as abscissa. Fig. 1 shows that this method gives a straight line, which can be extrapolated with greater accuracy than the curves which are obtained by application of the first or of the second method. In a recalculation of Sørensen's measurements of the osmotic pressures of concentrated solutions of albumin at the isoelectric point, we have found that the best approximation to a straight line is obtained by a fourth method, in which C_v, the protein concentration in g./100 cc. of solvent, is plotted as the abscissa and the ratio p/C_v is plotted as the ordinate.

All the methods described above yield a mean value for the molecular weight. In order to facilitate the comparison of different preparations, a slight modification of the general method has been devised, in which a value for the molecular weight in each experiment is calculated by formula (3)

$$p = \phi RT C_p = \phi RT\,[10C/M] \qquad \ldots\ldots(3),$$

C_p = g.-mols. of protein per litre of solution.

ϕ = a coefficient which represents the sum of the osmotic effects due to the ion pressure difference, the volume of the protein hydrate and the deviations from the ideal solution laws.

Theoretically, ϕ is equal to unity, if an infinitely dilute solution of the protein is equilibrated with a standard salt solution. At higher protein concentrations ϕ is equal to π_0/π, if M is constant.

The estimation of ϕ from the observed osmotic pressures may be simplified by the application of empirical formulae. If the pressures can be represented by formula (4) [Adair, 1928], the value of ϕ can be calculated by formula (6)

$$p = \frac{\pi_0 C}{1 - K_b C} \qquad \ldots\ldots(4).$$

K_b = an empirical constant. This constant has the same effect on the pressures as the volume correction "b" in van der Waals' equation. K_b represents the sum of the effects due to a number of factors, including the ion pressure difference.

Within the range of application of formula (4), there is a straight line relationship between $1/\pi$ and C, defined by formula (5)

$$\frac{1}{\pi} = \frac{1}{\pi_0} - \frac{K_b C}{\pi_0} \qquad \text{.....(5).}$$

The observations recorded in Fig. 1 show that a straight line is obtained in the case of solutions of serum albumin, if C is not greater than 5. The constant $1/\pi_0$ is equal to 0·422, the value of $1/\pi$ at $C = 0$, determined by extrapolation. The constant K_b can then be determined by applying the formula $K_b = \frac{\pi_0}{C}\left(\frac{1}{\pi_0} - \frac{1}{\pi}\right)$ to any point on the line where C is greater than 0. It will be observed that $1/\pi$ is equal to 0·287 when C is 5·0, and it follows that $K_b = 0{\cdot}064$.

Within the range of application of formula (5), the coefficient ϕ can be calculated by formula (6)

$$\phi = \frac{\pi}{\pi_0} = \frac{1}{1 - K_b C} \qquad \text{.....(6).}$$

$K_b = 0{\cdot}064$ in the case of serum albumin at p_H 7·4. In the case of serum globulin at the isoelectric point, $K_b = 0{\cdot}0282$.

The values of ϕ calculated by formula (6) and recorded in Table II must represent a mean value for all the experiments rather than the best value for any one preparation, and therefore the variation in the calculated molecular weights represents the sum of the variations in ϕ as well as the variations in M, but in spite of this source of error, the deviations from the mean value of $M = 71{,}980$ are within 4 %.

V. *The molecular weight of serum albumin.*

The first determination of the molecular weight of serum albumin in a system of well-defined composition is due to Sørensen [1925] who gave the figure 45,000. Adair [1926] obtained a higher value, 62,000. Svedberg and Sjögren [1928], investigating the problem by the ultra-centrifugal method, found that once crystallised serum albumin is a homogeneous substance of molecular weight 68,000, but stated that material three times crystallised was a mixture which contained decomposition products of low molecular weight. They therefore suggested that the proteins studied by Sørensen and by Adair were partially decomposed.

In this work, measurements of osmotic pressure have been made with serum albumin preparations crystallised once, twice and four times. The purity of the material was studied by the refractometric method described in a previous paper [Adair and Robinson, 1930]. The osmotic pressures given by each preparation are recorded in Table II. Fig. 1 shows that if $1/\pi$ be plotted as ordinate, and the concentration C as abscissa, a straight line is obtained over the range

from $C = 1{\cdot}0$ to $C = 5{\cdot}0$. By extrapolation it appears that $1/\pi = 0{\cdot}422$ when $C = 0$, and therefore $\pi_0 = 1/0{\cdot}422 = 2{\cdot}37$. By substitution in formula (2), the molecular weight at 1·0° is equal to 170,960/2·37 = 72,100.

In order to facilitate the comparison of the molecular weights in different experiments, calculations have been made by formulae (3) and (4) as described in the previous section. The results obtained with albumin prepared from fresh serum obtained from the Cambridge Field Laboratories and from Messrs Burroughs and Wellcome are recorded in Table II. It will be seen that the range of variation observed (from 69,200 to 74,900) is small, although some of the measurements have been made on material which has been crystallised four times. The estimations of the refraction increments given in a previous paper [Adair and Robinson, 1930], show that the preparations can be divided into two groups. In the first group, which includes preparations HA 1, 2, 3·2, 4·52, 4·0 and 4·5, attempts were made to remove lipins by washing the serum with ether or by recrystallisation. In these experiments, the refraction increment is about 0·00177, and the molecular weight varies from 70,100 to 74,900 (average 72,800). In the second group, which includes preparations HA 3·1, 3·3 and 4·1,

Table II. *Osmotic pressures and molecular weights of crystalline horse-serum albumin, equilibrated at 1·0° with a phosphate buffer mixture,* p_H *7·4.*

$\frac{0{\cdot}8}{15}M\ Na_2HPO_4 + \frac{0{\cdot}2}{15}M\ KH_2PO_4$.

Preparation No.	Exp. No.	C protein g./100 cc.	p pressure mm. Hg	π ratio p/C	ϕ correction formula (6)	Mol. weight	No. of crystallisations
HA 1·0*	2	2·08	5·58	2·68	1·15	73,700	1
HA 2·0*	5	4·86	16·76	3·45	1·45	72,100	1
,,	6	3·54	10·70	3·03	1·29	73,100	1
,,	7	2·49	6·87	2·76	1·19	73,700	1
,,	9	1·07	2·69	2·52	1·07	72,800	1
HA 3·1	12	2·19	6·25	2·85	1·17	69,900	1
,,	13	1·94	5·24	2·70	1·14	72,500	1
,,	14	4·64	16·00	3·45	1·42	70,600	1
,,	15*a*	3·66	11·65	3·18	1·31	70,400	1
HA 3·2*	16	2·83	8·50	3·00	1·22	73,800	1
,,	17	2·99	8·80	2·95	1·24	71,600	1
,,	18	1·50	4·02	2·68	1·11	70,600	1
,,	19	1·48	3·83	2·58	1·11	73,300	1
HA 3·3	20	3·62	11·52	3·19	1·30	69,900	1
,,	21*a*	1·96	5·46	2·79	1·14	70,200	1
,,	22*a*	1·97	5·18	2·63	1·14	74,400	1
HA 4·52	23	2·05	5·74	2·81	1·15	70,100	2
HA 4·00	24	1·86	5·00	2·68	1·14	72,400	4
,,	25	1·96	5·12	2·61	1·14	74,900	4
,,	26	3·87	11·96	3·09	1·33	73,600	4
,,	27	3·81	11·88	3·12	1·32	72,600	4
HA 4·5	28	1·58	4·25	2·69	1·11	70,700	4
,,	29	1·60	4·19	2·63	1·12	72,500	4
,,	30	2·82	8·37	2·97	1·22	70,200	4
,,	31	3·13	9·69	3·10	1·25	69,200	4
HA 4·1	34	4·61	15·40	3·34	1·42	72,700	1
,,	35	2·44	6·88	2·82	1·19	71,900	1

* Serum washed with ether.

no attempt was made to remove lipins. The refraction increments are about 3 % greater than the normal value, and the molecular weights range from 69,200 to 74,400 (average 71,200). If the lipins exert any osmotic pressure, the molecular weight of the protein calculated from the uncorrected pressure must be too small. The agreement between the figures quoted above shows that the lipins present in the impure preparations exert no appreciable osmotic pressure.

It may be observed that in three preparations, HA 3·1, 3·2 and 3·3, about half of the yield of crystals was dissolved by washing with a diluted solution of ammonium sulphate. According to Sørensen [1925], crystalline serum albumin can be separated into three fractions which have different solubilities but identical osmotic pressures. The partial fractionation effected in the preparations 3·1, 3·2 and 3·3 caused no significant deviation from the mean molecular weight.

The determinations of the molecular weight in Table II show that the results obtained by Svedberg and Sjögren are more accurate than the earlier figures given by Sørensen and by Adair, but Svedberg's suggestion that the earlier experiments were made with decomposed proteins is not supported by the experiments made in this work, which prove that the protein is not necessarily decomposed by the process of recrystallisation. It is probable that the low values of 45,000 given by Sørensen and 62,000 given by Adair are due to the approximations made in their provisional calculations. Adair [1928] published a revised estimate of 68,000 a short time before the appearance of Svedberg's paper.

In this work the methods for the determination of the molecular weight by extrapolation have been improved, as stated in Section IV, and it appears that in the case of Sørensen's measurements on albumin solutions containing 5 and 10 % of ammonium sulphate, the most probable values for the molecular weights are 76,000 and 72,000 respectively. The degree of accuracy of these figures is uncertain, because Sørensen gives no data for solutions containing less than 8 % of protein. They are about 60 % above the figure 45,000 calculated by Sørensen himself, but it should be observed that he states most emphatically that his result is a mere estimate of the order of magnitude. His calculation should give correct results in an ideal colloidal solution of the type described by Donnan, but errors of 60 % or more are by no means improbable in colloidal solutions of finite concentration.

In addition to the measurements on crystalline horse-serum albumin recorded in Table II, a number of preliminary determinations were made with other forms of albumin. The percentage of nitrogen in these proteins is not known; provisional estimates of the protein concentration have been made by assuming that, like crystalline horse-serum albumin, they contain 15·60 % of nitrogen.

The conalbumin of horse-serum, prepared from the mother liquor after the crystals have been removed, is probably a mixture of proteins. The average

molecular weight of this mixture (80,000 $\pm$ 8000) is greater than that of the crystalline fraction.

Rough estimates of the molecular weight of the sheep-serum albumin and of ox-serum albumin have been made by formula (3) on the assumption that ϕ is the same for all of the albumins at the same protein concentration, and it seems that, within the limits of experimental error, the size of the particles is the same in each species.

Molecular weights of about 70,000 were obtained in the case of both the total albumin of the sheep-serum and the fraction obtained in the form of an amorphous precipitate when acetic acid was added to a solution of sheep-serum albumin half-saturated with ammonium sulphate.

It has been found that the p_H can be reduced to 6·1 and the salt content increased to 1·0 equiv. without altering the molecular weight. Svedberg and Sjögren [1930] have stated that serum albumin is stable over the range p_H 4–9.

VI. *The molecular weight of serum globulin.*

A number of determinations of the molecular weight of globulin have been made by previous workers; Du Nouy [1924] obtained the figure 35,000 by applying his theory of the surface tensions of protein solutions; Sørensen [1925], Adair [1926] and Svedberg and Sjögren [1928], have obtained much larger values. Even if no corrections are made for deviations from the ideal solution laws in measurements of osmotic pressure, Sørensen's estimates range from 80,000 to 140,000. The corrections increase the estimates by about 60 % in the case of serum albumin, and it is probable that the estimates for globulin should be increased. The measurements required for making these corrections are not available.

In the systems investigated by Adair [1926], the molecular weight of euglobulin was about 174,000, and pseudoglobulin varied from 130,000 to 150,000, when the proteins were equilibrated at 0°, with $M/15$ phosphate buffer mixtures at p_H 7·4 and p_H 7·8.

Svedberg and Sjögren [1928] obtained the value 103,800 $\pm$ 3000, in the case of solutions of globulin in $\frac{1}{5}$ molar phosphate buffer mixture at p_H 5·5, centrifuged at temperatures of 18–26°. They state that although "total globulin" prepared by their method is a homogeneous substance of molecular weight 103,800, eu- and pseudo-globulins are mixtures of decomposition products, and they suggest that the results of previous workers were affected by the decomposition of the protein. In a recent paper, Svedberg and Sjögren [1930] state that the molecular weight of pseudoglobulin varies from 53,200 to 99,300. A further examination of the problem by osmometric methods has been undertaken in this work.

In order to minimise the possibility of errors due to changes in the protein, a number of preparations of total globulin were made by a rapid method, described by Svedberg and Sjögren [1928] and by Adair and Robinson [1930]. Although the protein is not perfectly stable under all conditions, the investiga-

tions referred to in Section III indicate that the degree of stability appears to be sufficient for the purpose of osmometric determinations at 0°.

Fig. 2 and Table III record observations of the osmotic pressure of unfractionated horse-serum globulin, equilibrated for periods of 2–12 weeks with the phosphate buffer mixture at the isoelectric point used by Svedberg. It will be seen that the variations from the mean pressure are comparatively small.

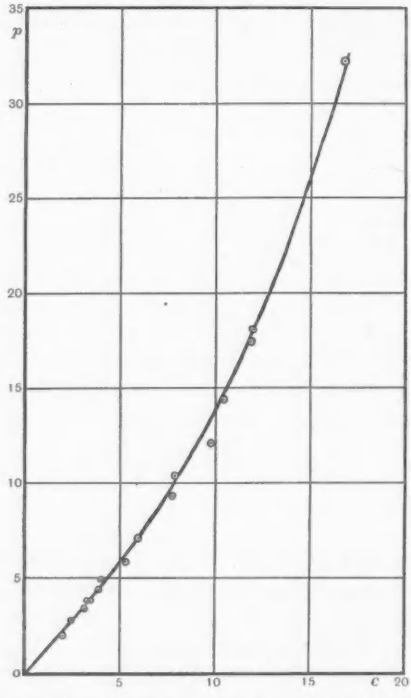

Fig. 2. Osmotic pressure of unfractionated serum globulin (horse) at p_H 5·35. Ordinate = pressure in mm. mercury at 1·0°. Abscissa = C = protein concentration in g. per 100 cc. solution. Curve calculated from formula 4.

Two methods for the preparation of euglobulin have been investigated. In the first method, which gave irregular values for the molecular weight, the euglobulin was precipitated by dilution and acidification of serum. Hardy [1905] has shown that the degree of dispersion of euglobulin prepared by this method is variable, and in this work it was found that the solutions remained opalescent even after the addition of salts at the physiological hydrogen ion concentration, and that the osmotic pressures were irregular. In the case of 2 % solutions, the pressures ranged from 1 to 2 mm. at p_H 7·4. In later experiments, a rapid method was adopted, in which the euglobulin was precipitated from fresh serum (diluted with an equal volume of water) by $\frac{1}{3}$

saturation with ammonium sulphate. The results obtained by this method are shown in Fig. 3.

Table III. *Osmotic pressures of isoelectric solutions of horse-serum globulin (unfractionated).*

Solvent 0·19 *M* KH_2PO_4 + 0·009 *M* Na_2HPO_4. p_H 5·35 at 1·0°.

Preparation No.	Exp. No.	*C*	*p*	π	ϕ	Mol. weight
2*	G 1	3·30	3·78	1·15	1·10	163,000
2*	G 2	3·39	3·84	1·14	1·10	165,000
1	G 3	4·02	4·96	1·24	1·12	154,000
3	G 6	5·33	5·86	1·10	1·17	181,000
3	G 7	16·98	32·28	1·90	1·92	172,000
3	G 9	11·92	17·51	1·47	1·50	174,000
3	G 10	9·84	12·24	1·24	1·38	189,000
3	G 11	7·74	9·32	1·20	1·27	180,000
3*a*	G 12*a*	2·04	1·92	0·94	1·06	192,000
3*a*	G 13*a*	3·15	3·44	1·09	1·09	171,000
4	G 16	12·00	18·11	1·51	1·51	170,000
4	G 17	7·93	10·42	1·31	1·28	167,000
4	G 18	5·98	7·09	1·19	1·20	171,000
4	G 19	3·92	4·39	1·12	1·12	171,000
4	G 20	4·01	4·53	1·13	1·13	169,000
4	G 21	10·45	14·40	1·38	1·42	174,000

* Serum treated with ether.
Preparations 1 and 2 = Serum from University Field Laboratory.
Preparations 3 and 4 = Serum from Burroughs and Wellcome.
Preparation 3*a* = Protein stored for 8 months.
$\phi = 1/(1 - 0{\cdot}0282C)$ formula (6).

Pseudoglobulin was obtained by half-saturation with ammonium sulphate. The results were rather variable, as shown by the data recorded in Fig. 3.

Before the molecular weight of a protein can be determined by the methods of extrapolation referred to in Section IV, it is necessary to prove that the degree of aggregation is independent of the protein concentration. Marrack and Hewitt [1929] suggest that the degree of aggregation of globulin is variable. A number of investigations have been made in this work which show that under certain conditions the observed osmotic pressures of globulin follow the laws which are characteristic of a protein of constant molecular weight, namely haemoglobin [Adair, 1928].

(*a*) In dilute solutions, containing less than 4 % of protein, the pressure is practically proportional to the protein concentration if the reaction is not too far from the isoelectric point, as shown by the data in Figs. 2 and 3.

(*b*) The pressure-concentration ratio π is increased by an increase in the protein concentration at the isoelectric point. According to the aggregation theory π should be diminished.

(*c*) The relationship between the pressures and concentrations in Fig. 2 is determined by a simplified form of van der Waals' equation $p\,(v - b) = RT$, which is applicable to solutions of haemoglobin at the isoelectric point, over a similar range of protein concentrations. Since v is equal to $1/C_p$, van der Waals' formula is equivalent to the expression $p = RTC_p/(1 - bC_p)$. If the protein concentration is given in g./100 cc., this equation may be restated in the form given in equation (4), $p = \pi_0 C/(1 - K_b C)$.

The determination of π_0 and K_b is described in Section IV. In the case of globulin, the results obtained are 1·0 and 0·0282 respectively. It will be seen that the line in Fig. 2, calculated by formula (4), is in agreement with the observed points. The constant K_b is equal to $100\,M\,b$, where b is van der Waals' constant, expressed in cc./g.-mol. It follows that if $K_b = 0{\cdot}0282$, there is a volume correction of 2·82 cc. per g. of globulin; in the case of haemoglobin,

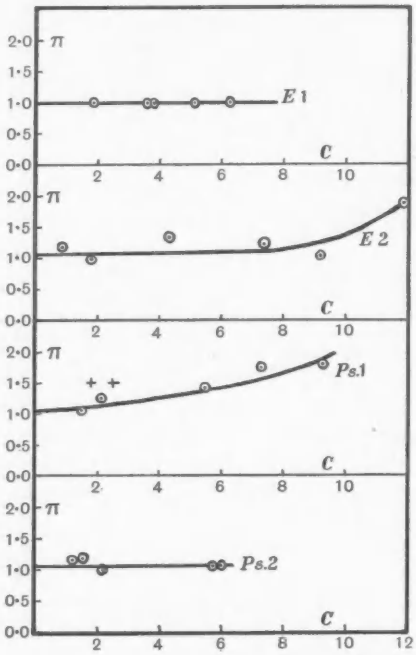

Fig. 3. Pressure-concentration ratios for fractionated globulin. Curves *E* 1 and *E* 2, euglobulin (sheep). Curve *Ps* 1, pseudoglobulin (sheep). Curve *Ps* 2, pseudoglobulin (ox). Solvent for *E* 1, *E* 2 and *Ps* 1, $M/10$ KCl + 0·95/15 M Na_2HPO_4 + 0·05/15 M KH_2PO_4, p_H 8·0. Solvent for *Ps* 2, 0·8/15 M Na_2HPO_4 + 0·2/15 M KH_2PO_4, p_H 7·4. Ordinate = π (formula 2). Abscissa = C, the protein concentration in g. per 100 cc. of solution.

K_b is about 0·026, and the volume correction is 2·6 cc. According to van der Waals, the value of b is four times the volume of the molecules. It is probable that the volumes of the hydrated globulin and haemoglobin are of the order of 1·3 and 0·96 cc./g. respectively [Weber and Nachmannsohn, 1929; Adair, 1928]. The volume corrections given above are less than four times the volume of the molecules, but there are many factors which might diminish the observed values of b.

The calculations in Table III show that the molecular weight of globulin in concentrated solutions is constant within the limits of error, if it is assumed

that the correction coefficient ϕ in formula (3) is determined by the formula $\phi = 1/(1 - 0{\cdot}0282C)$.

(*d*) Supplementary measurements indicate that the molecular weight of globulin remains constant over an appreciable range of concentrations of hydrogen ions and salts. Measurements of osmotic pressure of the same preparation of horse-serum globulin (No. 2) at p_H 5·35 and p_H 7·4 are recorded in Table III and Fig. 6. In the case of solutions containing 9 g./100 cc. of pseudoglobulin, at p_H 7·8, the pressures were 16·1, 16·5 and 18·2 mm. Hg respectively, when the concentrations of diffusible salts were 0·06, 0·23 and 1·12 g.-equiv. per litre of solvent. The changes in pressure caused by salts are relatively small, and it is almost certain that they are due to changes in the osmotic coefficient of the protein [Adair, 1928].

It is difficult to test the effects of temperature on the degree of aggregation of globulin by direct osmometric methods, because the purified protein is rather unstable at temperatures exceeding 0°, but according to Marrack and Hewitt [1927] it appears that variations from 0° to 37° do not alter the state of aggregation of the mixture of albumin and globulin present in serum. Svedberg finds no change in the molecular weight over the range 18–26°.

On the whole, the evidence in favour of the hypothesis that the molecular weight is constant appears to be sufficient to justify the estimation of the ratio π_0 and the calculation of the molecular weight of globulin from the experiments recorded in Figs. 2 and 3, although it is not inconceivable that a combination of the effects of changes in aggregation and repulsive forces between the particles might produce the effects predicted for a system in which the molecular weight is a constant.

A summary of the provisional calculations is given in Table IV. It will be seen that the molecular weight of euglobulin prepared by the second method described above is practically the same as the molecular weight of total globulin. The preparation of pseudoglobulin is a longer process and the lower values obtained may be due to decomposition, as suggested by Svedberg. The absolute values of the molecular weight are 60–70 % greater than Svedberg's value 103,800; at present it is not possible to explain this difference.

Table IV. *Provisional estimations of the molecular weight of globulin dissolved in phosphate mixtures at 0°.*

No. of determinations	Fraction	p_H	Mol. weight	Notes
13	Total globulin	5·35	175,000 ± 17,000	Horse-serum*
3	,,	5·35	163,000 ± 9,000	Horse-serum†
2	,,	7·4	163,000 ± 4,000	,,
1	,,	7·4	174,000	Ox-serum
1	,,	7·4	160,000	Sheep-serum
4	Euglobulin	7·8	170,000 ± 10,000	,,
6	,,	7·8	162,000 ± 20,000	,,
10	Pseudoglobulin	7·8	155,000 ± 47,000	,,
5	,,	7·4	150,000 ± 20,000	Ox-serum

* Burroughs and Wellcome. † Field Laboratory.

VII. *The relationships between fractionated albumins and globulins and the proteins of natural serum.*

Since the work of Hardy [1905] on the globulins, it has been recognised that the separation of serum into fractions may be correlated with changes in the state of aggregation of the proteins. According to Moore and Roaf [1907] the process of separation increases the aggregation of the proteins, but the opposite view that the albumins and globulins are fractions of a single protein has received more support [Starling, 1926]. Sørensen [1925] has suggested that complexes of euglobulin and pseudoglobulin may be formed.

In this work, the state of aggregation of the proteins in unfractionated serum has been studied by comparing the observed osmotic pressure of the unfractioned protein with the theoretical pressure p, calculated by applying Dalton's Law of partial pressures, as stated below

$$p = p_i + p_a + p_g \qquad \text{.....(7).}$$

p_i = the ion-pressure difference, p_a and p_g the partial pressures of albumin and of globulin. The calculation of p_i is described in Section IX. The estimation of p_a and p_g is exceedingly difficult in the case of normal serum, which contains about 8 % of protein, on account of the interionic forces, and until the effects of these forces have been subjected to more detailed investigation, it is necessary to work with more dilute solutions. In the limiting case of an infinitely dilute solution of the protein, the term p_i is zero and the pressure is determined by the formula:

$$p = RT\,[10\,C_a]/M_a + RT\,[10\,C_g]/M_g \qquad \text{.....(8).}$$

C_a and C_g = g. of albumin and globulin per 100 cc. of diluted serum. M_a and M_g denote the molecular weights of albumin and globulin in serum. In applying this formula it has been assumed that $M_a = 72{,}000$ and $M_g = 170{,}000$. The theoretical value of the ratio π_0 calculated by formula (8) is equal to $2{\cdot}36\,x_a + 1{\cdot}00\,(1 - x_a)$, where x_a is equal to g. of albumin per g. of serum protein, and hence $1 - x_a$ is equal to the g. of globulin per g. of serum protein. The values of x_a for horse-serum and for ox-serum, given in Table V, are the averages of the results obtained by Bächer and Kosian [1924]; the value for human serum is that given by Marrack and Hewitt [1927].

The observed values of π_0 for horse-serum and ox-serum have been obtained by applying the method illustrated in Fig. 1 to the experiments recorded in Tables VI and VII. We have attempted to estimate the value of π_0 for human serum protein from the data published by previous workers, including Verney [1926], Mayrs [1926] and Marrack and Hewitt [1927]. The results obtained from their measurements are $1{\cdot}8 \pm 0{\cdot}2$, $1{\cdot}89 \pm 0{\cdot}2$ and $1{\cdot}9 \pm 0{\cdot}2$ respectively. In all cases the pressures have been recalculated in mm. Hg and reduced to 0°.

The results summarised in Table V show that the observed and the theoretical values of π_0 agree within the limits of experimental error. The theo-

retical values are 2–5 % higher than those observed, but differences of this order might be accounted for if the proportion of globulin in the actual specimens used were slightly higher than usual. Moreover, it appears that a part of the albumin designated "conalbumin" may have a molecular weight greater than 72,000 (Section V).

Since the observed and the theoretical values of π_0 agree in the case of sera of three different species, it seems justifiable to infer firstly that the methods used in this work cause little or no change in the state of aggregation of albumin and globulin and secondly that the proteins in diluted serum are a mixture rather than a compound of albumins and globulins. The osmotic pressures of concentrated solutions of serum protein are represented in Fig. 7, and the form of the curves obtained is consistent with the hypothesis that no change in aggregation takes place. The osmotic pressures of undiluted sera with different proportions of albumin have been studied by Govaerts [1925, 1927], who states that his formula is empirical, and without any physical significance. It may be noted that in his formula the ion pressure difference p_i has been neglected. The results recorded below show that p_i is relatively large in undiluted sera.

Table V. *Application of Dalton's law of partial pressures to dilute solutions of unfractionated serum proteins.*

Species	x_a albumin g./g. of total protein	π_0 theoretical formula (8)	π_0 observed
Horse	0·525	1·72	1·64±0·1
Ox	0·507	1·69	1·63±0·1
Man	0·688	1·94	1·90±0·2

Table VI. *Osmotic pressure of unfractionated ox-serum proteins.*

Outer fluid $\frac{0·8}{15}$ *M* Na_2HPO_4 + $\frac{0·2}{15}$ *M* KH_2PO_4 at 0°.

Exp. No.	C	C_v	p	π	E_m	E_m calculated by formula $E_m = 0·141 C_v$
32	0·96	0·97	1·2	1·25	−0·15	−0·14
33	1·16	1·17	2·2	1·90	—	−0·165
28	2·10	2·15	3·7	1·76	—	−0·303
29	2·17	2·21	3·9	1·80	−0·3	−0·312
27	3·71	3·85	7·6	2·05	−0·75	−0·543
26	4·31	4·50	8·4	1·95	−0·50	−0·635
23	8·30	9·06	20·9	2·52	−1·30	−1·28
22	8·62	9·45	22·4	2·59	−1·05	−1·33
31	15·37	18·15	59·1	3·85	−2·20	−2·56
30	16·75	20·10	72·9	4·35	−2·90	−2·83

Table VII. *Osmotic pressure of unfractionated horse-serum proteins.*

Outer fluid $\frac{0·2}{15}$ *M* KH_2PO_4. + $\frac{0·8}{15}$ *M* Na_2HPO_4. at 0°.

Exp. No.	C	p	π
1	9·82	24·2	2·46
3	4·9	9·9	2·02
4	4·93	9·55	1·93
5	2·14	4·05	1·89
6	2·11	3·52	1·67

VIII. *The conditions for the calculation of the ion pressure difference by Donnan's formulae.*

In ideal solutions, the ion pressure difference symbolised p_i must be determined by the formula of Donnan [1911]. In a later paper Donnan and Allmand [1914] have given formulae for the activities of the ions applicable to non-ideal solutions, but Donnan's treatment of osmotic pressures refers to infinitely dilute solutions. It has been suggested [Hecht, 1925; Marrack and Hewitt, 1927], that Donnan's formulae can be applied to the osmotic pressures of the serum proteins, but the experimental evidence is not conclusive, because the observed pressures are affected by an unknown factor, the osmotic pressure of the protein ions themselves. It is possible to investigate the problem without making any assumptions concerning the partial pressures of the protein ions, if determinations of the distribution of the diffusible anions and cations are correlated with electrometric measurements of the membrane potentials.

The results obtained by the equilibration of ammonium albuminate and an ammonium phosphate buffer mixture at p_H 7·3 are recorded in Tables VIII and IX. The experiments show that the ammonium albuminate is ionised, and that there is an excess of diffusible ions inside the membrane. If the system is within the range of application of Donnan's equations, the pressure p_i should be determined by formula (9)

$$p_i = RT\{[NH_4]_c' + [H_2PO_4]_c' + [HPO_4]_c' - [NH_4]'' - [H_2PO_4]'' - [HPO_4]''\} \quad \text{.....(9),}$$

$[NH_4]''$, $[H_2PO_4]''$ and $[HPO_4]''$ denote the concentrations of the ions in g.-mols./litre of dialysate.

$[NH_4]_c'$, $[H_2PO_4]_c'$ and $[HPO_4]_c'$ denote the corrected concentrations of the ions in the protein solution. A correction is necessary, as stated by Sørensen [1917], on account of the volume occupied by the protein referred to below. According to Donnan's theory, a measurement of the distribution ratio for one of the ions makes it possible to calculate the ratios for all the ions. If the ratio $[NH_4]_c'/[NH_4]''$ is equal to λ, the distribution ratios for H_2PO_4' and HPO_4'' ions are λ^{-1} and λ^{-2} respectively. These ratios are correlated with the membrane potential by formula (11). It follows that if the concentrations of all the ions in the dialysate are known, their concentrations in the protein solution can be calculated by a number of different methods.

In the first place the corrected[1] concentration of each ion can be calculated by formula (10)

$$m_i' = c_i' \times \frac{100}{100 - V_1C} \quad \text{.....(10),}$$

m_i' = corrected concentration of the ion in the protein solution, in g.-mols./litre of "solvent." c_i' = concentration in g. mols./litre of solution, determined by chemical analysis. V_1C = % of the total volume occupied by protein.

[1] This method of correction has been used instead of the calculation of the molalities of the ions, in g.-mols./kg. of water, because it facilitates the estimation of the partial pressures of the protein ions.

C = g. protein per 100 cc. solution. V = effective volume occupied by 1 g. of protein plus the water combined with it. According to Weber and Nachmannsohn [1929] $V_1 = 1{\cdot}0$ cc. in the case of serum albumin.

The data given in Table VIII show that the sum of the corrected concentrations in experiment SA 42 is 0·1989. The sum of the concentrations in the dialysate is 0·185. The value of p_i calculated from the difference is $17{,}033 \times 0{\cdot}0139 = 236$ mm. Hg. In experiment SA 43, p_i is approximately 311 mm.

In the second method the corrected concentrations of the ions are calculated from measurements of the membrane potential by formula (11), a slight modification of Donnan's equation for the membrane potential

$$\log m_i' = \log c_i'' - n_i FE/2{\cdot}303\,RT \qquad \text{.....(11).}$$

E = the membrane potential in volts. n_i = the valence of the diffusible ion (negative for anions). $2{\cdot}303\,RT/F$ = a constant = 0·05416 at 0°. c_i'' = g.-mols. of the diffusible ion per litre of dialysate.

The measurements in Table VIII show that the "ideal values" of m_i' calculated by this formula are smaller than the corresponding values calculated by formula (10). The differences show that the protein must diminish the "activity coefficient" of the diffusible cations by about 4 %. In the case of the anions the diminution is about 12 %. Similar changes have been observed by Hastings, Salvesen, Sendroy and Van Slyke [1927] and by Adair [1928].

If the ion pressure difference is calculated by applying formula (9) to the

Table VIII. *Sheep-serum albumin, equilibrated with* $\frac{0{\cdot}75}{15} M\ (NH_4)_2HPO_4 + \frac{0{\cdot}25}{15} M\ NH_4H_2PO_4$, p_H *7·3 at 0°.*

Exp. No.	SA 42	SA 43	SA 44
p osmotic pressure mm. Hg	56·5	54·6	53·3
C g. protein per 100 cc. solution	10·09	10·00	10·18
C_v g. protein per 100 cc. solvent	11·22	11·11	11·35
Density of inner fluid at 0°	1·0337	1·0326	1·0334
Density of outer fluid at 0°	1·0057	1·0055	1·0062
E_m = membrane potential millivolts	−2·25	−2·55	−2·35
p_H of outer fluid minus p_H of inner fluid	0·0416	0·0470	0·0435
$[NH_4]''$ outer, g.-mols./litre	0·1183	0·1145	0·1172
$[NH_4]'$ inner, g.-mols./litre	0·1214	0·1213	0·1311
$[PO_4]''$ outer, g.-mols./litre	0·0667	0·0669	0·0663
$[PO_4]'$ inner, g.-mols./litre	0·0575	0·0582	0·0567
*Ratio $\frac{HPO_4''}{\text{total } PO_4}$ outer	0·775	0·712	0·768
*Ratio $\frac{HPO_4'}{\text{total } PO_4}$ inner	0·753	0·689	0·736
$[NH_4]_c'$ observed [Formula (10)]	0·135	0·135	0·145
$[NH_4]_c'$ ideal [Formula (11)]	0·130	0·128	0·130
Ratio $\frac{[NH_4]_c \text{ ideal}}{[NH_4]_c \text{ observed}}$	0·96	0·95	—
$[PO_4]_c'$ observed	0·639	0·647	0·632
$[PO_4]_c'$ ideal	0·563	0·557	0·556
Ratio $\frac{[PO_4]_c' \text{ ideal}}{[PO_4]_c' \text{ observed}}$	0·882	0·861	0·896

* Ratio $HPO_4/HPO_4 + H_2PO_4$ = fraction of total phosphate in bivalent form, calculated by correlating the direct measurements of the ratio for the outer fluid and the membrane potentials by formula (11).

"ideal values" of m_i' recorded in Table VIII the result obtained is 29 ± 3 mm. In addition to the results obtained by methods 1 and 2, described above, calculations have been made by other methods and are summarised in Table IX. It will be seen that the results vary from 1·4 to 273 mm. The range of variation is large in comparison with the differences between the ideal and the observed concentrations, because small errors in the theoretical assumptions are exaggerated in the calculation of p_i which represents a small difference between two relatively large pressures. A number of modifications of formula (9) have been suggested [Loeb, 1922; Hecht, 1925; Marrack and Hewitt, 1927], but the degree of accuracy of the assumptions involved is rather uncertain.

Table IX. *Calculations of the ion pressure difference by Donnan's formulae.*

Sheep-serum albumin equilibrated at 0° with $M/15$ ammonium phosphate buffer, p_H 7·3.

Method	Calculated p_i
Observed excess of ions inside membrane	273 ± 37
Ammonia distribution ratio	60
Membrane potential	29 ± 3
Equivalents ammonium proteinate	15 ± 4
Total phosphate distribution ratio	1·4

IX. *The calculation of the ion pressure difference in non-ideal solutions.*

In systems where the deviations from the ideal solution laws are not great, it is possible to estimate the value of p_i by a simple modification of Donnan's theory [Adair, 1928]. The deviations shown in Table X are so large that it is necessary to adopt an entirely different method of treatment in order to obtain a formula for the ion pressure difference. The derivation of the more accurate formula (12) has been given in a previous paper [Adair, 1929, 2]

$$p_i = RT \int_0^u m_p n_p du \qquad \text{.....(12).}$$

m_p = g.-mols. of protein per litre of solvent [*cf.* formula (13)]. n_p = the mean valence of the protein ions. The symbol u is defined as the value of $E \times F/RT$, where E, the membrane potential, is a function of the protein concentration, determined under conditions where the protein concentration is varied from 0 to m_p, and the activities of water and of the diffusible salts are kept constant by the equilibration of the protein solutions, enclosed in membranes, with a solution of electrolytes of constant temperature, pressure and composition.

Materials required for the application of the formula to horse-serum albumin have been summarised in Table X. It will be observed that there are slight variations in the refractive index and in the p_H of the outer fluids, due to the evaporation of water and to traces of alkali from the glass and other factors, but the variations in the osmotic pressures and membrane potentials due to these changes are within the limits of experimental error.

Table X. *Osmotic pressures and membrane potentials of crystalline horse-serum albumin.*

Outer fluid $\frac{0{\cdot}8}{15}M$ $Na_2HPO_4 + \frac{0{\cdot}2}{15}M$ KH_2PO_4 at 0°.

Preparation No.	Exp. No.	C	$\sum C_v$	p	π	E_m membrane potential in millivolts	p_H outer fluid final value	Difference between refractive indices of outer fluid and of water
HA 4·1	32	9·14	10·05	45·8	5·01	−2·0	7·46	0·00168
,,	33	6·60	7·07	28·2	4·27	−1·55	7·45	0·00168
,,	34	4·60	4·82	15·38	3·34	−1·10	7·55	0·00167
,,	35	2·44	2·50	6·88	2·82	−0·73	7·49	0·00170
,,	36	7·41	8·00	31·15	4·20	−1·85	7·49	0·00170
HA 5	37	9·97	11·09	60·2	6·04	−2·65	7·41	0·00173
,,	38	6·65	7·12	30·3	4·56	−1·72	7·43	0·00170
,,	39	5·64	5·98	25·6	4·54	−1·50	7·42	0·00171
,,	40	3·84	3·99	13·9	3·61	−1·10	7·40	0·00170
,,	41	2·39	2·45	7·5	3·13	−0·73	7·40	0·00174

The values of C_v given in this table are defined by formula (13) (*cf.* formula (10)).

$$C_v = C \times \frac{100}{100 - V_1 C} \qquad \text{.....(13),}$$

C_v = g. dry protein per 100 cc. solvent.

If the molecular weight M is known, the corrected concentration m_p can be calculated by the formula $m_p = 10 \times C_v/M$.

The determination of the valence n_p and the equivalent concentration of the protein ions $n_p m_p$ requires further consideration. Theoretically, $n_p m_p$ must be equal to the difference between the equivalent concentrations of the diffusible anions and of the diffusible cations. The determination of the concentrations of the ions by chemical methods is open to criticism, on the ground that a certain proportion of the ions may be bound by the protein. Electrometric measurements are not affected by the bound ions but the result may be difficult to interpret on account of the effects of interionic forces. The effects of these forces can be represented by the activity coefficient f_i', defined by the formula $a_i' = m_i' f_i'$, where a_i' represents the activity of the diffusible ions in the protein solution. The activities of hydrogen and of chloride ions can be determined by direct electrometric methods, but if direct methods are not available calculations can be made by the thermodynamical formula of Donnan and Allmand [1914], restated below

$$\log m_i' f_i' = \log c_i'' f_i'' - n_i EF/2{\cdot}303\, RT \qquad \text{.....(14).}$$

f_i'' = the activity coefficient of the diffusible ion in the dialysate.

If f_i' is equal to f_i'', and if E is less than 0·002, formula (14) can be replaced by the series formula $m_i' = c_i''[1 - n_i u + \frac{1}{2} n_i^2 u^2]$, and under these conditions, $m_p n_p$ is determined by formula (15). Similar formulae have been referred to by Bjerrum [1924], by Rinde [1926] and by Adair [1928]

$$m_p n_p = uJ \qquad \text{.....(15).}$$

J = the ional concentration, or the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valences.

Formula (15) may be subject to considerable errors if the solutions are not ideal, but it has been observed that the difference between f_i' and f_i'' diminishes as the protein concentration is diminished, if the concentration of salts in the dialysate is kept constant [Adair, 1928]. According to formula (14) the effects due to the interionic forces cancel out, if $f_i' = f_i''$. Provisionally therefore it seems reasonable to assume that formula (15) can be applied to the limiting values of the ratio $(E_m/C_v)_0$ defined by formula (16)

$$n_p = \frac{uJ}{m_v} = 0{\cdot}00425\,M\,J\,(E_m/C_v)_0 \qquad \text{.....(16).}$$

0·00425 is a constant equal to $(F/10{,}000\,RT)$ at 0°. M = the molecular weight of the protein (72,000). E_m = the membrane potential in millivolts = $E \times 1000$, C_v = g. dry protein per 100 cc. of solvent. $(E_m/C_v)_0$ = the limiting value of the ratio (E_m/C_v) at $C_v = 0$, determined by extrapolation. The curve in Fig. 4 indicated that the limiting value of this ratio is about $0{\cdot}324 \pm 0{\cdot}1$. This ratio indicates that $n_p = -34{\cdot}3$, in the case of a very dilute solution of serum albumin equilibrated with the phosphate buffer at p_H 7·4.

The calculation of the valence in more concentrated protein solutions requires further consideration, because an alteration of the protein concentration from $C_v = 0$ to $C_v = 12$ is correlated with a change in the membrane potential and a diminution of the p_H of the protein solution from 7·4 to 7·35. It has been shown that the amount of base bound by serum albumin is proportional to the observed p_H minus 4·8 [Cohn, 1925] and it follows that n_p may be diminished by about 2 %. This change is within the limits of experimental error.

After the values of u, m_p and n_p have been determined, formula (12) can be integrated by graphical methods, but in many cases, the calculation can be shortened by the application of an empirical formula, of the type stated below

$$m_p n_p = Ju + Jku^2 \qquad \text{.....(17),}$$

k = an empirical constant. Within the limits of experimental error the measurements in Fig. 4 are represented by the curve calculated by the empirical formula $(C_v/E_m) = 3{\cdot}09 + 0{\cdot}7\,E_m$; it follows that k is about 5·3.

Within the range of application of formula (17) the ion pressure difference is determined by formula (18)

$$p_i = RT \int_0^u J\,(u + ku^2)\,du = RTJ\,\tfrac{1}{2}u^2 + RTJk\,\tfrac{1}{3}u^2 \qquad \text{.....(18).}$$

If the pressures are expressed in mm. Hg at 0°, formula (18) is equivalent to formula (19)

$$p_i = 15{\cdot}4\,JE_m^2 + 0{\cdot}436\,JkE_m^3 \qquad \text{.....(19).}$$

In the system referred to in Table X, J is equal to 0·3466, and k is approximately 5·3 and it follows that $p_i = 5{\cdot}35\,E_m^2 + 2{\cdot}32\,E_m^3$. A number of calculations of p_i made by this formula are recorded in Table XI. In this table

the partial pressure of the protein ions, symbolised p_p, has been calculated from the observed osmotic pressure p by the formula $p_p = p - p_i$. The osmotic

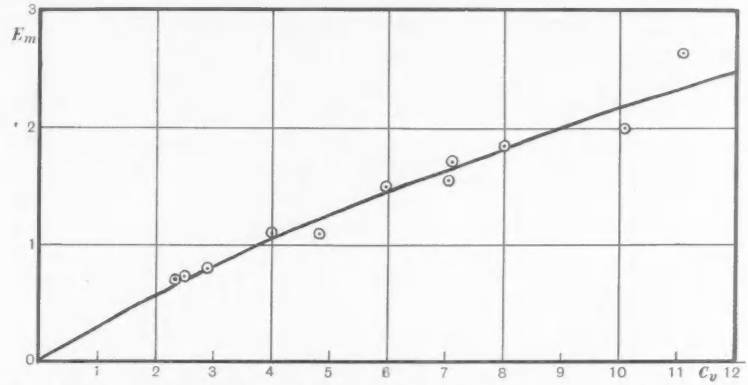

Fig. 4. Membrane potentials of crystalline serum albumin at p_H 7·4. Ordinate = membrane potential in millivolts at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. solvent. Curve calculated from formula $C_v = 3{\cdot}09\,E_m + 0{\cdot}7\,E_m^2$.

coefficient g_p, defined by the formula $p_p = RTg_p m_p$, has been given in the last column of Table XI. It will be observed that in fairly dilute solutions in which the concentration m_p is less than 0·0005, g.-mols./litre of solvent, the osmotic coefficient diminishes as the protein concentration is increased, in accordance

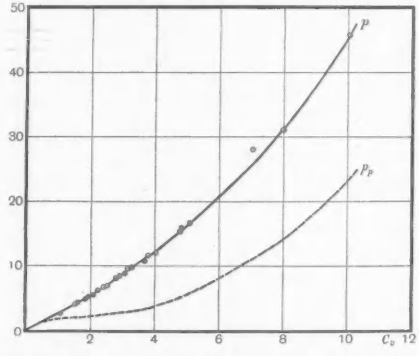

Fig. 5. Partial osmotic pressure of crystalline serum albumin at p_H 7·4. Ordinate = pressure in mm. mercury at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. solvent. p = observed osmotic pressure. $p_p = p - p_i$ = partial pressure due to protein ions.

with the interionic attraction theory of Debye and Hückel [1923]. In more concentrated solutions, the effects of the electrical forces described by Debye and Hückel are masked by other factors and the osmotic coefficients tend to increase. According to their theory, the interionic forces depend upon the squares of the valences of the ions; although their equations cannot be applied to

protein systems without modification, it is interesting to note that there is a marked difference between serum albumin (valence − 37 at p_H 7·4) and haemoglobin (valence − 7 at p_H 7·8), in accordance with the theory. In the case of haemoglobin, the effects of the interionic forces are masked by other factors even in dilute solutions, and the osmotic coefficients and the activity coefficients exceed unity at all protein concentrations [Adair, 1927, 1928, 1929, 1].

Table XI. *The ion pressure difference in solutions of crystalline horse-serum albumin equilibrated with* $\frac{0{\cdot}8}{15}M$ $Na_2HPO_4 + \frac{0{\cdot}2}{15}M$ KH_2PO_4. p_H 7·4 *at* 0°.

C_v g. protein per 100 cc. solvent	m_p g.-mols protein per litre solvent	E_m membrane potential millivolts	p observed osmotic pressure mm. Hg	p_i ion pressure difference	p_p partial pressure of protein ions	RTm_p ideal value of p_p	g_p osmotic coefficient p_p/RTm_p
1·7	0·00024	−0·5	4·4	2·2	2·2	4·1	0·54
3·8	0·00053	−1·0	11·2	7·6	3·6	9·0	0·40
6·2	0·00086	−1·5	22·4	13·2	9·2	14·7	0·63
9·0	0·00125	−2·0	38·0	19·9	18·1	21·2	0·85
10·8	0·00150	−2·3	50·5	24·6	25·9	25·5	1·01

Theoretically, the osmotic coefficients of the ions of globulin at p_H 7·4 should be intermediate between the values obtained for albumin and for haemoglobin, if the valence of the globulin has an intermediate value. The provisional estimates recorded in Fig. 6 are in agreement with this hypothesis.

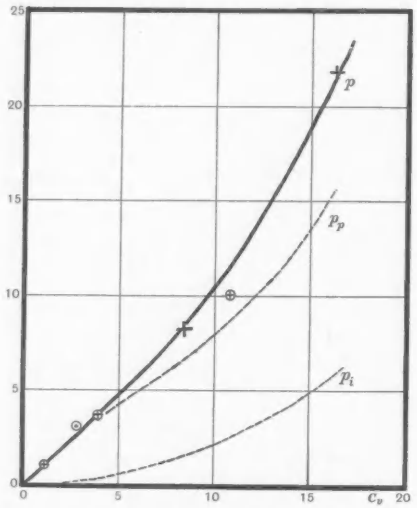

Fig. 6. Partial osmotic pressure of unfractionated globulin at p_H 7·4. Ordinate pressure in mm. mercury at 0°. Abscissa $= C_v =$ corrected concentration of protein in g. per 100 cc. of solvent (formula 10). $p =$ observed osmotic pressure; $p_p =$ partial pressure of protein ions; $p_i =$ diffusible ion pressure difference. Crosses = globulin of sheep. Circles = globulin of ox, except point at $C_v = 2{\cdot}8$, which represents 2 observations on preparation *G* 2 (horse).

Hardy [1905] did not detect any ionic globulin in serum by the method of kataphoresis. Preliminary measurements of the membrane potentials indicate that the ratio (E_m/C_v) is about 0·065 ± 0·025, in the case of globulin at p_H 7·4, and from this result it appears that the valence of the ions may be about − 16·3, a value which is not large in comparison with the size of the molecule. It is possible that this low ratio of the charge to the mass and the effects of the ion atmosphere, described by Debye, prevent any appreciable movement in the electric field.

A series of 8 measurements of the membrane potentials of the total proteins from ox-serum has been recorded in Table VI. It is possible that the ratio E_m/C_v is a function of the protein concentration as in the case of serum albumin, but as a first approximation, the observed values of the membrane potential may be represented by the empirical formula $E_m = 0{\cdot}141\ C_v$ in which the coefficient 0·141 is the mean value of the ratio E_m/C_v over the whole range of protein concentrations. Approximate values of p_i at different protein concentrations have been calculated by formula (20)

$$p_i = 15{\cdot}4\ JE_m^2 \qquad \text{.....(20).}$$

Since $J = 0{\cdot}3466$, $p_i = 5{\cdot}33\ (0{\cdot}141\ C_v)^2$. The results are recorded in Fig. 7.

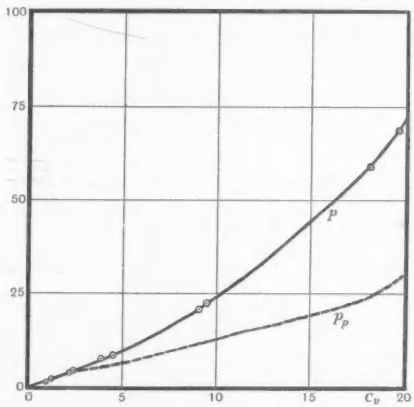

Fig. 7. Osmotic pressure of unfractionated proteins of ox-serum at p_H 7·4. Ordinate = pressure in mm. mercury at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. of solvent (calculated from formula 10). p = observed osmotic pressure. $p_p = p - p_i$ = partial pressure due to protein ions.

This curve is of interest in connection with the theories advanced by previous workers concerning the high pressures of concentrated solutions of serum proteins which have been observed by Starling [1899] by Krogh and Nakazawa [1927] and other workers. Verney [1926] has suggested that the effect may be due to the hydration of the protein. Marrack and Hewitt [1927] have suggested that the effect may be accounted for by the ion pressure difference. Their hypothesis is confirmed by the results recorded in Fig. 7. The

curve for the partial pressure p_p is a much closer approximation to a straight line than is the case with pure serum albumin or haemoglobin. It is probable that the apparent approximation to a straight line in concentrated solutions is partly accidental. It may be due to errors in the estimation of the ion pressure difference, or it may be due to a balancing of the effects of attractive and repulsive forces which may take place in certain ranges of protein concentrations.

Summary.

The stability of the serum proteins has been examined by a number of methods, and it has been found that serum albumin can be recrystallised four times without change in the molecular weight. The preparations of albumin made in this work appear to be more stable than those made by Svedberg and Sjögren, who state that three crystallisations lead to decomposition of albumin.

The methods for the determination of molecular weights of proteins in non-ideal solutions [Adair, 1924, 1928] have been developed, with special reference to the estimation of the molecular weight of serum proteins in the physiological range of hydrogen ion and salt concentrations.

The mean value of the molecular weight of horse-serum albumin in 27 experiments has been determined as 72,000 $\pm$ 3000. One preparation gave a value of 67,000. Sørensen's provisional estimate of 45,000 is subject to a correction of about 60 % on account of deviations from the ideal solution laws. The corrected value (about 74,000) agrees with the results obtained in this work. Preliminary determinations indicate that the serum albumins of the ox and sheep have molecular weights of about 70,000.

The mean value of the molecular weight of the unfractionated globulin of the horse is 175,000. In a series of 17 experiments the maximum and minimum values observed were 192,000 and 154,000 respectively. The unfractionated globulins of the ox and the sheep gave similar results. Euglobulin, prepared by a rapid method, resembles total globulin. Svedberg's value for total globulin, 103,800, is lower than that obtained in this work. The factors which might account for the difference have been studied, but at present no explanation can be offered.

The osmotic pressure of the total protein of serum before fractionation has been studied from the point of view of Dalton's law of partial pressures, and it appears that serum protein is not a compound of albumins and globulins. The state of aggregation of the proteins in the untreated serum appears to be the same as their state of aggregation in the purified proteins prepared by the methods used in this work.

The protein solutions which have been examined are beyond the range of application of Donnan's formulae for osmotic pressures. Attempts to estimate the pressure due to the excess of diffusible ions inside the membrane (p_i) by these formulae yield contradictory results.

Methods for the estimation of p_i in non-ideal solutions are described, and calculations of p_i and of p_p, the partial pressure of the protein ions, have been made. The application of the theory of Debye and Hückel to these partial pressures has been discussed.

A method has been suggested for the estimation of the valence of the protein ions in non-ideal solutions.

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CCXIII. THE INHIBITION OF ESTERASES BY EXCESS SUBSTRATE.

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(*Received November 3rd, 1930.*)

It is now widely though not universally accepted that the first stage in enzymic hydrolysis consists of a reversible one-to-one union of enzyme and substrate, this complex later breaking down to give free enzyme once more and the products of the reaction. On this view Michaelis and Menten [1913] built up their equation giving the relation between velocity of reaction and substrate concentration, a relation which is similar to that between the degree of dissociation of a weak acid and the hydrogen ion concentration. That is, the relation is expressed by a rectangular hyperbola, or, if the concentration be plotted on a logarithmic scale, the familiar S-shaped curve is obtained. On either system of plotting, at high substrate concentration the curve should run parallel to the x-axis, the velocity being unaffected once the enzyme is saturated. Actually, Michaelis and Menten found in their experiments with invertase that the velocity began to diminish again when a concentration of about 6 % was exceeded, but they concluded that this might be due to failure of the laws of dilute solutions, the change in the concentration of water becoming an appreciable factor. The work of Nelson and Schubert [1928] subsequently showed that this assumption was both justified and, to a first approximation, quantitatively sufficient.

With other enzymes, however, the velocity passes a maximum and falls again at much smaller concentrations. Particularly with the liver esterases is this the case. Furthermore, the concentration at which this occurs varies very markedly with the species from which the enzyme is obtained [Bamann and Schmeller, 1929; Murray and King, 1930]. Thus with sheep-liver esterase hydrolysing ethyl butyrate the maximum velocity is observed when the substrate is at about 0·005 M concentration, with rabbit-liver esterase when it is at more than 0·01 M. Obviously another explanation must be sought for this phenomenon.

Haldane [1930] has suggested that the explanation might lie in the formation of a complex of the enzyme with two molecules of the substrate, this complex being incapable of the breakdown leading to liberation of acid and alcohol. This idea leads to the following extension of the original Michaelis-Menten equation. Three reactions are to be considered, (1) $E + S \rightleftharpoons ES$,

(2) $ES + S \rightleftharpoons ES_2$, and (3) $ES \rightarrow E + P$. Let s, θ, p, q, be the concentrations of substrate, free enzyme, ES, and ES_2, σ the total enzyme concentration, K_1, K_2, the equilibrium constants of equations (1) and (2) and k the velocity constant of equation (3). Then $\theta s = K_1 p$ and $ps = K_2 q$, so that

$$\sigma = \theta + p + q = \theta\left(1 + \frac{s}{K_1} + \frac{s^2}{K_1 K_2}\right).$$

But $$V = kp = k \cdot \frac{s}{K_1} \cdot \theta = k\sigma \frac{s}{K_1 + s + \frac{s^2}{K_2}} \quad \text{or} \quad k\sigma \frac{1}{1 + \frac{K_1}{s} + \frac{s}{K_2}}.$$

It will be seen that this expression is similar in form to that obtained by Michaelis [1911] for the dissociation residue of an ampholyte, *i.e.*

$$\rho = \frac{1}{1 + \frac{K_a}{[H^\cdot]} + \frac{K_b}{[OH']}},$$

if for [OH'] is written $\frac{K_w}{[H^\cdot]}$. Haldane's suggestion thus calls for an activity-p_s curve symmetrical about the highest point and of the familiar bell-shaped type.

Some of the curves given by Bamann and Schmeller approximate to this type, but the number of observations is too small to form the basis of a theory. In the present work therefore the curves have been extended into more concentrated solutions, and the effect of competitive inhibitors has been studied and compared with that predicted on theoretical grounds on the basis of the assumption described.

The activity-p_s *curve and the determination of* K_1 *and* K_2.

The procedure followed is that of Murray and King [1930], with certain additions as described in the experimental section. Sheep-liver esterase was the enzyme chosen, since for this the optimum concentration falls about the middle of the logarithmic scale of practicable values. Ethyl butyrate was the substrate. The general symmetry of the activity-p_s curve was quickly apparent, so that the next step was to calculate the best representative values of K_1 and K_2 and plot the theoretical curve with a view to observing how the experimental points lay with respect to it.

The product $K_1 K_2$ was determined as follows. By virtue of the symmetry of the curve, for all pairs of points with equal y co-ordinates (velocity) the sum of the x co-ordinates (log. concentration) must be equal. But $s = K_1$, $s = K_2$, are such a pair of points, so that this sum equals $\log K_1 + \log K_2$ or $\log K_1 K_2$. Hence a mean value of $K_1 K_2$ can be obtained directly from the experimental points.

The following method was devised to calculate the quotient K_1/K_2: transfer the y-axis to the axis of symmetry of the curve, that is, to the point $s = \sqrt{K_1 K_2}$, so that $x = \log s - \log \sqrt{K_1 K_2}$. Therefore

$$s = e^{(x + \log \sqrt{K_1 K_2})} = \sqrt{K_1 K_2}\, e^x.$$

Therefore

$$y = \frac{\text{const.}}{1+\frac{K_1}{s}+\frac{s}{K_2}} = \frac{\text{const.}}{1+\frac{K_1}{\sqrt{K_1K_2}}e^{-x}+\frac{\sqrt{K_1K_2}}{K_2}e^{x}} = \frac{\text{const.}}{1+\sqrt{\frac{K_1}{K_2}}(e^{x}+e^{-x})} = \frac{\text{const.}}{1+2\sqrt{\frac{K_1}{K_2}}\cosh x}.$$

Before using this equation, the values of x, if given in ordinary logarithms, must be multiplied by $\log_e 10$ (2·303), since the values required for the equation are Napierian logarithms.

A mean value of K_1/K_2 was found by taking a number of pairs of points and eliminating the arbitrary constant of the numerator.

The best representative values of the product and of the quotient were as follows:

$$K_1K_2 = \text{antilog } \bar{5}\cdot03 = 0\cdot0000107, \quad K_1/K_2 = 0\cdot1137,$$

whence

$$K_1 = 0\cdot00114 \text{ and } K_2 = 0\cdot00971.$$

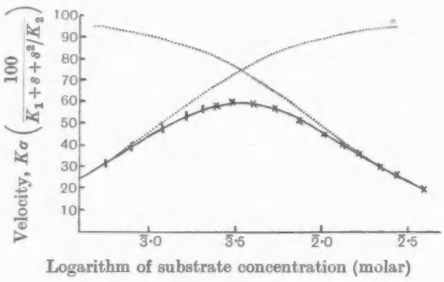

Fig. 1.

The continuous curve in Fig. 1 is the curve obtained by plotting the equation

$$y = \frac{100}{1+\frac{0\cdot00114}{s}+\frac{s}{0\cdot00971}}.$$

The dotted curves are the simple Michaelis curves

$$y = \frac{100}{1+\frac{0\cdot00114}{s}} \text{ and } y = \frac{100}{1+\frac{s}{0\cdot00971}}.$$

The points are the experimentally observed velocities, the scale being chosen so that one standard point (0·00517 M or 0·48 mg./cc.) falls on the curve as drawn.

The agreement of the points on the right-hand side of the curve is as good as is to be expected, while on the left-hand side, where the velocities required (the initial velocities) have to be obtained by extrapolating backwards, with a consequent increased margin of error, the "theoretical" velocity is always within the limits of the experimental values.

The ordinate of the graph represents the percentage of the limiting velocity, $k\sigma$, which would be reached if the whole of the enzyme could be "saturated" in the ordinary Michaelis sense, that is, if the combination with the second substrate molecule did not occur. It will be seen that even in the optimal conditions only 60 % of that activity is reached. This point will be more fully considered in the next Section.

The effect of competitive inhibitors.

The satisfactory agreement between the observed velocities and those calculated on Haldane's assumption led next to an extension of the equations to a system containing a competitive inhibitor. The additional equilibrium $E + I \rightleftharpoons EI$ has to be considered[1]. If h = concentration of inhibitor, r = concentration of EI, the extra relationship is $\theta h = K_h r$. Then

$$\sigma = \theta + p + q + r, \quad \sigma = \theta\left(1+\frac{s}{K_1}+\frac{s^2}{K_1K_2}+\frac{h}{K_h}\right),$$

$$V = k.\frac{s}{K_1}.\theta = k\sigma\frac{\frac{s}{K_1}}{\frac{K_1}{K_1}+\frac{s}{K_1}+\frac{s^2}{K_2}+\frac{hK_1}{K_h}} = k\sigma\frac{1}{1+\frac{K_1}{s}\left(1+\frac{h}{K_h}\right)+\frac{s}{K_2}}.$$

This is equivalent to a change in the first affinity constant but not in the second. A curve of the same family is obtained, but the left-hand side is pushed in. In such a family the maximum value of the ordinate depends on the quotient K_1/K_2, just as in the case of the ampholyte [Michaelis, 1911] the percentage capable of existing as a dissociation residue depends on the product $K_a K_b$.

Certain quite definite differences from the case of an enzyme such as invertase are thus to be expected if this theory is correct. In the invertase system the same ultimate maximum velocity is obtained with or without inhibitor, the presence of inhibitor merely necessitating a greater substrate concentration before the saturating concentration is reached. In this case the addition of inhibitor affects the actual maximum velocity obtainable, diminishing it as well as pushing it into the direction of higher substrate concentration. If sufficient of the inhibitor be present, the enzyme can be completely inactivated whatever the concentration of substrate.

From already published results of Murray and King [1930] it is evident that this is what actually occurs in the case of the inhibitor methyl-*n*-hexylcarbinol. Fig. 1 of that paper is expressed with the concentration on the ordinary scale, which brings it out less clearly than the logarithmic, but shows clearly enough that the optimum substrate concentration is raised while the maximum velocity reached is lowered. Fig. 2 of this paper shows the effect (freshly determined with certain corrections as described in the experimental part) with the concentration plotted logarithmically.

[1] Haldane has worked out the case, considering also the reactions $EI + I \rightleftharpoons EI_2$ and $EI + S \rightleftharpoons ESI \rightleftharpoons ES + I$. The coefficient of the $1/s$ term is thereby altered by a different extent, the coefficient of s still remains unaltered, *viz.* $1/K_2$. Only the quantitative, not the qualitative, effect is thus altered by these extra considerations.

An attempt was made to substitute simpler substances for the rather complex secondary alcohols employed in the previous work. With the four primary alcohols, methyl to *n*-butyl, however, it was found that the simple relation

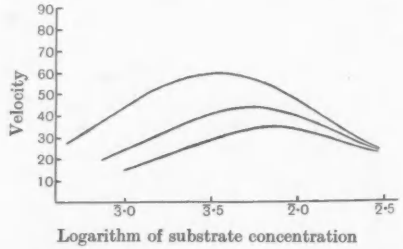

Fig. 2. Velocity-p_s curve in presence of (i) 0·00016 M, (ii) 0·00032 M *d*-methyl-*n*-hexyl carbinol.

deduced did not hold. Superimposed on the competitive inhibition which produced a shift of the position of the maximum velocity point towards higher concentration was a non-competitive inhibition which resulted in a depression of the curve over its whole length. In other words, a part of the inhibition produced was independent of the substrate concentration, though a part apparently was competitive. Fig. 3 shows the effect of *n*-butyl alcohol. The

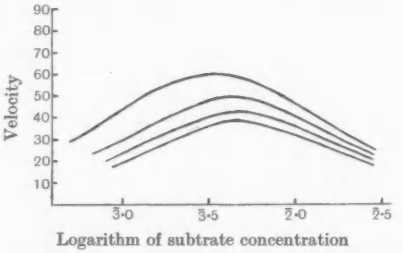

Fig. 3. Velocity-p_s curve in presence of (i) 0·0034 M, (ii) 0·0068 M, (iii) 0·0102 M *n*-butyl alcohol.

secondary alcohols, on the other hand, were true competitive inhibitors, and in high substrate concentrations the inhibition was extremely small and tending to zero.

The results with those inhibitors which are strictly competitive therefore further confirms the suggestion of Haldane. It is not necessary here to discuss the exact significance or nature of the ES_2 complex. Haldane himself has suggested that the most likely interpretation is that for hydrolysis to take place the substrate has to be united to the enzyme by two points, one characteristic of the acid part of the molecule, one of the alcohol. Then, if the acid end of one molecule and the alcohol end of another become attached to the same enzyme

molecule, the strain on the molecules leading to decomposition would not be produced. Diagrammatically the idea is expressed:

Capable of breakdown Incapable of breakdown

At the present time there is not sufficient evidence to go further than merely to suggest this view.

Experimental.

As the technique used was described in the previous paper [Murray and King, 1930], attention will be drawn to only a few points, the importance of which has been realised later, and in particular to two sources of error which affect the quantitative though not the qualitative findings.

The first of these is connected with the use of alcoholic solutions of indicators. Although only three drops of indicator were added to the 8 cc. of experimental fluid, controls showed that this produced an inhibition. As explained before, ethyl alcohol inhibits both competitively and non-competitively, so that the whole length of the curve was depressed, but especially so the low substrate concentration end. At 0·005 M concentration of substrate (near to the optimum) the inhibition was about 15 % and was greater at higher dilution. Further controls showed that the indicator itself, if made up as an aqueous solution of its sodium salt, had no inhibitory properties, and subsequently it was always used in this form.

The second error was due to dissolved carbon dioxide. The amount dissolved in the reagents could be roughly gauged by the number of drops of alkali required to adjust the p_H before the experiment began. Whenever this was more than about 3 drops or 0·075 cc. of N/100, the left-hand side of the curve was found to be depressed, although the high concentration figures were hardly affected. Thus the sodium bicarbonate formed appeared to act as a competitive inhibitor. Whether this could be due to its carbonyl group is an interesting question. The competitive nature of the inhibition was further demonstrated by repeatedly shaking the contents of the tube with air to dissolve more CO_2 and then neutralising with N/100 NaOH; in this way increasing inhibitions were produced. It was not possible to add any considerable quantity of $NaHCO_3$, since its buffering properties prevent its use in this essentially buffer-free technique.

Previous to this finding the stock solution of the sparingly soluble ethyl butyrate had been made by shaking violently with water in the presence of air, the resulting fluid being then neutralised, so that it contained some bicarbonate. Subsequently the shaking was done in the absence of air, the drops being broken up by small pieces of glass rod. Owing to ethyl butyrate being lighter than water the bottle had to be filled and inverted under cold boiled water and the ethyl butyrate introduced by pipette, the pieces of glass being pushed

in from beneath before the stopper was inserted. This procedure, though complicated, gave the most satisfactory CO_2-free solution of the substrate.

The effect of these two discoveries was to show that the maximum of the curve was considerably more to the left than had been at first supposed. Thus, whereas this was given as 0·005 *M* before, when these sources of inhibition were removed 0·003 *M* was nearer to the mark. Bamann and Schmeller [1929] found the maximum for methyl butyrate at still higher concentration, *viz.*, nearly 0·01 *M*. Their system was very possibly already partially inhibited in this or another way. As these workers state, the value found for the affinity should always be qualified by the epithet "apparent," since the effect of a competitive inhibitor is to alter the value of the constant which is taken to be the measure of the affinity.

SUMMARY.

The suggestion of Haldane, that the inhibition of certain enzymes by excess of substrate is due to the formation of a non-hydrolysable complex of the enzyme with two molecules of the substrate, is tested for the esterase of sheep-liver and found to explain the facts satisfactorily and to predict correctly the effect of competitive inhibitors.

The work described was performed during the tenure of the Benn W. Levy Studentship, to the managers of which I wish to express my thanks. I am particularly grateful to Prof. J. B. S. Haldane for informing me of his views before they appeared in print, and for suggesting simplifications in the mathematical treatment. To Sir F. G. Hopkins I express my thanks for his continued interest and encouragement.

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CCXIV. INVESTIGATIONS ON THE SEPARATION OF THE DIGESTION PRODUCTS OF THE PROTEINS BY THE METHOD OF DIFFERENTIAL DIALYSIS.

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Before Hofmeister [1902] enunciated what we now refer to as the peptide hypothesis, researches into the structure of the proteins had been confined almost entirely to attempts to characterise the proteoses and peptones which arise from them by the action of digestive enzymes or of acids and alkalis. This earlier work is now almost forgotten and will probably never be of more than historical interest, for the products obtained by tedious methods of fractionation were not what would be regarded to-day as definite chemical compounds. The peptide hypothesis caused the interest of chemists to swing from the more complex products of protein degradation to the final products, the amino-acids, and researches since that date have been concerned chiefly with the characterisation of these simple products and with the synthesis from them of more complex bodies of known chemical constitution.

Until quite recently it seemed that synthetic polypeptides furnished a type model of the proteins sufficiently accurate to account for most of their chemical characteristics, but with increasing knowledge of polypeptide structure has come increasing knowledge of the proteins themselves, especially of their physical character and of the way in which they are attacked by the proteolytic enzymes. The view that linkages other than the peptide linkage may play an important part in protein structure is growing, and it is probable that before long chemists will again have to turn their attention to the earlier products of degradation of proteins—products which are far more complex than any synthetic polypeptide, and which still retain some of the unique characters of the proteins themselves. The late Prof. Schryver, realising that the older salting-out methods of separation were too tedious and gave products too heavily contaminated with inorganic material to be of use for this purpose, suggested that an easier and more reproducible separation could be obtained by the differential dialysis of the digestion products through membranes of known permeability.

The present research on these lines is to be regarded as a preliminary investigation, for the quantities of material which have been dealt with were small. Sufficient evidence has been adduced however to show that a fractionation of the more complex products of protein digests is possible, and that the method may be of service in any future attempt to isolate and study the composition and properties of proteoses and peptones.

Experimental.

Membranes of graded permeability were made by the method of Pierce [1927], using ethylene glycol as a non-volatile water-soluble swelling agent. A 2 % solution of collodion instead of the 1 % recommended by Pierce was used, as this was found to give membranes which were strong enough to withstand unaided the pressure of the dialysing solution.

5 g. of dry nitrocellulose were placed in a 250 cc. measuring flask and 100 cc. of anhydrous alcohol-ether mixture (1 : 3) were added. After the nitrocellulose had passed into solution (2 days), varying quantities of specially dried ethylene glycol were added, followed by anhydrous alcohol-ether mixture to make 250 cc. The solution was well shaken and kept for use.

A plate of smooth glass of uniform thickness was first well cleaned and dried with a soft cloth. Ether-alcohol mixture was then poured over the surface and wiped off with dry filter paper so as to remove all traces of moisture and fine dirt. The cleaned plate was floated on mercury lying in a Petri dish which had been placed in a $CaCl_2$ desiccator. A ring or frame 9·6 × 6·0 cm. (previously dried in an oven at 100°) was placed on the floating plate, and 5 cc. of the collodion solution were poured over them from a dry pipette. The desiccator was quickly closed, and dry air was drawn through the apparatus at a uniform rate for 24 hours. A sulphuric acid trap attached to the exit tube effectively prevented the back entry of moisture. At the end of this time the membrane, still attached to the glass plate, was immersed in water. It then peeled off the plate quite easily and was washed in a stream of running distilled water for 48 hours. To obviate any differences in thickness the dialysing portion of the membrane was confined to the central 6·25 × 2·5 cm. only. The composition of the membranes is indicated in Table I; they became clogged if used more than once, and a fresh membrane was therefore employed for each experiment.

Table I. *Composition of the membranes for the dialysis of protein digests.*

No.	Nitrocellulose g.	Ethylene glycol cc.	Alcohol-ether mixture cc.	Nitrocellulose/water ratio
4·0	2	2·0	98·0	1 : 0·996
4·25	2	2·5	97·5	1 : 1·247
4·50	2	3·0	97·0	1 : 1·502
4·75	2	3·5	96·5	1 : 1·763
5·0	2	4·0	96·0	1 : 2·043

The dialysing cells were specially prepared from hollowed out ebonite blocks and had internal dimensions of 6·5 × 2·8 × 1·2 cm. The membranes

were placed between rubber washers and were then clamped one each side of the dialysing cell by means of ebonite rings which could be drawn towards each other by means of long bolts. The stirring which is essential for rapid dialysis was accomplished by passing in a steady stream of washed sterile air. In case of frothing liquids a few drops of capryl alcohol were put in the cells and the air was passed through bubblers containing capryl alcohol. The arrangement was found to be simple and effective.

These cells were completely immersed in distilled water in a specially constructed continuous dialysis apparatus, similar in design to that used by Schryver [1908], wherein the dialysate was continuously withdrawn and replaced by distilled water.

The first series of membranes prepared had nitrocellulose/water ratios ranging from 1 : 0·003 to 1 : 8·24. These were first tried on simple substances such as sodium chloride, ammonium molybdate, glucose, soluble starch, etc. and were found to have a satisfactory selective permeability. The first product of a protein nature to be examined was a "bactopeptone" prepared from meat, the fractional dialysis of which is given in Table II. The amino-N of each

Table II. *Fractional dialysis of bactopeptone.*

(Figures given are in percentages of total nitrogen.)

Membrane No. (1)	Nitrocellulose/ water ratio (2)	Total N in the successive fractions obtained (3)	Amino-N (4)	Peptide-N (5)	Non-amino-N (6)
2	1 : 0·003	2·8	2·5	0·2	0·0
3	1 : 0·219	2·8	1·2	0·3	1·3
4	1 : 0·510	39·9	9·1	21·7	9·0
5	1 : 2·083	20·7	3·9	11·0	5·7
6	1 : 4·102	28·9	5·1	11·1	12·5
7	1 : 8·237	5·25	1·2	0·8	0·7

Table III. *Fractionation of bactopeptone by salt precipitation.*

Fraction		% of total N
(*A*) Primary proteoses	Precipitated by half saturation with Na_2SO_4 at 33°	13·8
(*B*) Secondary proteoses	Precipitated by full saturation of the filtrate from *A*	44·4
(*C*) Peptones	Precipitated by 14 % tannic acid at 20° at p_H 7 from filtrate of *B*	23·8
(*D*) Sub-peptones	Precipitated by 95 % alcohol from filtrate of *C* at p_H 5	12·4
(*E*) Simple peptides and amino-acids	Not precipitated by these reagents	5·5
	Total	100·0

fraction was determined by the micro-method of Van Slyke, and the peptide-N in a similar way after hydrolysis for 20 hours with an equal volume of concentrated hydrochloric acid. Non-amino-N was obtained by difference, and represents the N which was not in amino- or peptide-form.

To compare the fractionation thus obtained with that given by the older precipitation methods, another sample of the bactopeptone was fractionated

by a modification of the method of Wasteneys and Borsook [1924], as shown in Table III. The two series of fractions thus obtained are correlated in Table IV. Agreement is fair, showing that differential dialysis, which introduces no

Table IV. *Comparison of the fractionation of bactopeptone by dialysis and salt precipitation.*

(Figures given are percentages of total nitrogen of the bactopeptone.)

Fraction	Dialysis		Precipitation	
1	2·8	5·6	5·52	Amino-acids and simple peptides
2	2·8			
3	39·9		36·28	12·4 Sub-peptones 23·8 Peptones
4	20·7	49·5	44·4	Secondary proteoses
5	28·9			
6	5·3	5·9	13·80	Primary proteoses
7	0·7			

foreign material such as Na_2SO_4 or tannic acid, can be used for the preparation of proteoses and peptones.

After the detailed examination of the fractions of the commercial peptone, the study of the differential dialysis of enzymic hydrolysates of pure proteins was undertaken. The proteins selected for preparing the digests, *i.e.* gelatin, egg-albumin, blood-fibrin, glutelin and caseinogen, were those which could easily be purified and which would be representative of typical groups.

The standard method of preparing a pepsin digest was adopted. Pepsin scales were added to a 2 % solution or suspension of protein in 0·4 % hydrochloric acid and the mixture was incubated at 37° with further additions of pepsin until digestion was complete. Usually a period of 3 months was required for maximum hydrolysis. The digest so produced was neutralised with sodium hydroxide, and the solution was concentrated *in vacuo* below 45°. The concentrated solution was made up so as to give a 10 % solution of the protein digest, and small amounts of chloroform and toluene were added to keep it aseptic. The p_H of the digest remained unchanged during the proteolysis.

On account of possible hydrolysis the distribution of nitrogen in the dialysate was not determined directly. It was computed by difference from the initial and final amounts of the forms of nitrogen present in the dialysis cell.

Peptic digest of gelatin.

In the preparation of the digests Coignet's Gold Label gelatin was used. In the present case it took 67 days to reach the point when further addition of pepsin produced no increase in free amino-nitrogen. The distribution of nitrogen in the digest was as follows: amino-N, 26·9 %; peptide-N, 38·4 %; non-amino-N, 34·7 %. It is to be noted that Frankel [1916] did not obtain, with pepsin, protein digests containing more than 20 % of the total amino-N as free amino-N, and consequently came to the conclusion that pepsin hydrolyses only about 20 % of the amide linkages of the proteins. The free amino-nitrogen of

the present digest of pure gelatin shows that (if the enzyme is allowed to act for a sufficiently long time) more than 40 % of the available amino-nitrogen is set free.

It is interesting to compare the non-amino-nitrogen content of the digest with that given by acid hydrolysis. The most recent determination of the distribution of nitrogen in gelatin by a modification of Van Slyke's method [Narayana and Sreenivasaya, 1928] shows 15·87 % arginine, 2·74 % histidine, and 18·8 % non-amino-non-basic nitrogen. Calculating from these figures, the total amount of non-amino-nitrogen is 32·53 %, in fairly close agreement with the 34·7 % obtained in the present digest.

Table V. *Fractional dialysis of a peptic digest of gelatin.*

(Figures given are in percentages of total nitrogen of the digest.)

	Fractions	Total N	Amino-N	Peptide-N	Non-amino-N
	Original digest	100	26·9	38·4	34·7
1	Dialysing out of membrane No. 4	9·8	3·6	1·9	4·3
2	Not dialysing out of No. 4 but dialysing out of No. 4·25	15·3	8·4	0·1	6·9
3	Not dialysing out of No. 4·25 but dialysing out of No. 4·5	42·8	9·1	21·7	11·6
4	Not dialysing out of No. 4·5 but dialysing out of No. 4·75	20·9	3·7	10·2	7·1
5	Not dialysing out of No. 4·75 but dialysing out of No. 5	0·8	0·4	0·0	0·4
6	Not dialysing out of No. 5	10·8	1·7	4·7	4·4

The nitrogen distribution in the various fractions (Table V) shows, as one would expect, a diminishing amount of amino-N and an increasing amount of peptide-N with increasing molecular weight. The non-amino-N is fairly evenly distributed, and does not suggest that proline or hydroxyproline is localised in any inner nuclei.

Tryptic digest of gelatin.

The digest was prepared in the following way. Coignet's Gold Label gelatin was dissolved in warm water and the solution was diluted to 2 %. Sufficient Na_2CO_3 was then added to make 0·4 %. Trypsin (1 g. per 100 g. of gelatin) was added and the solution was incubated at 37°. Further small amounts of trypsin were added from time to time during the next three months until the digest showed no appreciable increase in free amino-nitrogen.

The distribution of nitrogen in the digest shows amino-nitrogen 30·6 %, peptide-nitrogen 15·0 %, and non-amino-nitrogen 54·5 %. Neither gelatin nor its peptic digest when completely hydrolysed yields more than 34 % of the nitrogen in non-amino-form, so that the distribution of nitrogen is altered in favour of the non-amino-form by the prolonged action of trypsin. This curious fact might suggest the existence in the gelatin molecule of bonds which are hydrolysed in a different manner by pepsin or acid and by trypsin, an observation which is of interest in view of recent work on the structure of the gelatin molecule carried out in this laboratory [*cf.* Knaggs and Schryver, 1924].

Table VI. *Fractional dialysis of a tryptic digest of gelatin.*

(Figures are in percentages of the total nitrogen of the digest.)

		Total N	Amino-N	Peptide-N	Non-Amino-N
	Digest	100	30·6	15·2	54·2
1	Dialysing out of No. 4	42·4	11·0	10·5	20·9
2	Not dialysing out of No. 4 but dialysing out of No. 4·25	51·6	18·7	1·3	31·6
3	Not dialysing out of No. 4·25	6·0	0·9	3·4	1·7

Alternatively the large amount of non-amino-nitrogen may be due to diketopiperazines which are stable to hydrolysis with 20 % HCl. It is interesting here to recall that Blanchetière [1928] has observed extensive formation of diketopiperazine rings in a tryptic digest of egg-albumin. Whether these rings existed in the original gelatin molecule, or whether they are secondary products, cannot be profitably discussed here on the scanty evidence available.

A discussion of the individual fractions of the differential dialysis brings out certain interesting points.

Fraction 1. The distribution of nitrogen in this fraction shows that it contains most of the peptide-nitrogen of the digest. The peptide-nitrogen is therefore present almost entirely in the form of simpler polypeptides, which could, presumably, be hydrolysed almost completely by erepsin.

Fraction 2. This fraction is nearly half of the digest and consists mainly of amino- and non-amino-nitrogen which are present in the low ratio of 1 : 2·5. The peptide-nitrogen is almost negligible indicating that simple polypeptides must be absent. Linkages which may or may not involve nitrogen must be present in the compounds of this fraction, for although these contain amino-acids as components, it is clear that the amino-groups are all free, and that the acids must be linked in some way through their carboxyl groups. It is possible that the trypsin has attacked peptide bonds in the outer region of the protein molecule, leaving nuclei composed of amino-acids or other substances united by linkages that may not be of peptide nature. Such nuclei might be composed chiefly of amino-acids which do not contain reactive amino-groups—suggesting the "arginine nuclei" of Kossel [1901] or the kyrines of Siegfried [1904].

Peptic digests of egg-albumin, blood-fibrin, glutelin and caseinogen.

Full analyses of these digests have been made, and a condensed summary of the results is given in Table VII. In each case the fractions were obtained by using membranes similar to those referred to in Table I. The fractions show an increasing ratio of peptide-N to amino-N. The non-amino-N which, in the case of each digest, is approximately equal to the value which can be calculated from the known amino-acid composition of the respective protein, shows irregularities which seem worth recording.

One final observation which deserves mention is the high degree of proteolysis brought about by pepsin in all of these digests. Frankel [1916] found that practically all proteins he examined were acted upon by pepsin to the same

extent. In the present research it has been found that under the prolonged action of pepsin each protein is hydrolysed to a different degree and the maximum proteolysis obtained is much more than that obtained by Frankel (Table VIII).

Table VII. *Showing the fractional dialysis of peptic digests of certain proteins.*

(Figures given in percentages of total nitrogen of the digests.)

	Egg-albumin				Blood-fibrin			
Fraction	Total N	Amino-N	Peptide-N	Non-amino-N	Total N	Amino-N	Peptide-N	Non-amino-N
(Peptone)	100	24·0	62·2	13·8	100	24·2	46·0	29·8
1	8·7	1·7	3·1	3·9	9·7	4·6	2·1	3·0
2	26·5	6·2	17·6	2·7	5·9	0·6	3·0	2·3
3	40·4	12·4	27·0	1·0	22·3	10·5	11·3	0·5
4	24·4	2·7	15·5	6·2	12·8	0·3	4·0	8·5
5	—	—	—	—	22·8	5·5	10·5	6·8
6	—	—	—	—	26·5	2·7	15·1	8·7

	Glutelin				Caseinogen			
	Total N	Amino-N	Peptide-N	Non-amino-N	Total N	Amino-N	Peptide-N	Non-amino-N
(Peptone)	100	29·9	32·1	38·0	100	26·2	44·6	29·2
1	26·5	8·2	7·2	11·1	23·2	4·4	11·2	7·6
2	1·4	2·1	0·0	− 0·7	8·3	4·0	0·7	3·6
3	47·5	14·6	13·4	19·5	8·3	1·9	—	6·5
4	1·3	1·1	0·2	0·0	48·9	13·8	25·6	9·5
5	3·3	1·2	0·3	1·8	3·3	0·9	0·9	0·5
6	21·0	2·7	11·0	7·3	8·0	1·2	6·3	1·5

Table VIII. *Total amino-nitrogen of various proteins liberated by pepsin.*

Protein	Approximate time of proteolysis (months)	% of amino-N
Gelatin	3	41·2
Egg-albumin	3	28·0
Fibrin	4	34·5
Glutelin	4	48·0
Caseinogen	3	37·0

SUMMARY.

A method of differential dialysis suitable for the investigation of the digestion products of the proteins has been worked out.

The dialysing membranes of graded permeability are prepared from nitrocellulose dissolved in an ether-alcohol mixture to which is added a suitable amount of ethylene glycol as a non-volatile water-soluble swelling agent [Pierce, 1927].

Peptic digests of gelatin, egg-albumin, blood-fibrin, glutelin and caseinogen as well as a tryptic digest of gelatin and "bactopeptone" have been fractionated by these membranes.

Fractions of increasing molecular complexity, as shown by estimations of free amino- and peptide-nitrogen, can be readily obtained, and the method

seems suitable for the preparation of these substances in amounts sufficient for more complete chemical analysis.

I have to thank the late Prof. S. B. Schryver for suggesting this research, and Dr A. B. P. Page, Dr K. V. Thimann and Dr H. W. Buston for their help and advice.

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CCXV. THE DEVELOPMENT AND PHOSPHATASE ACTIVITY *IN VIVO* AND *IN VITRO* OF THE MANDIBULAR SKELETAL TISSUE OF THE EMBRYONIC FOWL.

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Introduction.

In a previous communication [Fell and Robison, 1929] observations were recorded on the development and phosphatase activity of embryonic femora and limb-buds cultivated *in vitro*. It was found that the isolated femur of the 5–6 day embryonic fowl continued both its anatomical and histological development when explanted *in vitro*, underwent periosteal ossification and also synthesised a phosphatase which normally occurs in considerable quantities *in vivo* in bone and ossifying cartilage [Robison, 1923]. Explants of the 3-day mesenchymatous limb-bud, on the other hand, gave rise to cartilage of a different type which neither ossified nor synthesised phosphatase.

As a continuation of these earlier experiments it was decided to investigate the histology, development and phosphatase activity *in vivo* and *in vitro* of two other types of skeletal tissue: (*a*) cartilage which normally does not ossify, and (*b*)[1] membrane bone, *i.e.* bone which develops in a membrane of connective tissue and is not deposited on the surface of the cartilage as in the case of a so-called "cartilage-bone" such as the femur.

The lower jaw of the embryonic fowl provided admirable material for such a study. In a late fowl embryo the mandible consists of a rod of cartilage, known as Meckel's cartilage, which is surrounded by a sheath of membrane bone. Meckel's cartilage only ossifies in the articular region and the greater part of it persists, until the end of development, as an unossified cartilaginous rod.

The chief objects of the present study were as follows.

(1) To find whether any histological difference exists *in vivo* between non-ossifying cartilage such as the distal part of Meckel's rod and ossifying cartilage such as the palato-quadrate.

[1] These experiments were undertaken at the suggestion of Prof. J. T. Wilson.

(2) To determine whether the non-ossifying Meckel's cartilage synthesises phosphatase.

(3) To investigate the histological development and phosphatase activity of Meckel's cartilage when isolated from its normal environment and explanted *in vitro*.

(4) To compare the histological behaviour and phosphatase activity of normal and explanted mandibular osteogenic mesoderm.

Material and methods.

Material.

Fowl embryos and chicks were used throughout the investigation. The normal material examined was obtained from embryos and chicks of various ages up to the 13th day after hatching. The explants were taken from $5\frac{1}{2}$ and 6-day embryos.

Culture technique.

The cultures of Meckel's rods, palato-quadrates and femora were made by the watch-glass method described in our previous communication [Fell and Robison, 1929]. The culture vessels ($1\frac{1}{2}$ inch watch-glasses in 3 inch Petri dishes) were somewhat smaller than those used before, and the culture medium consisted of only four drops of embryo extract and four drops of plasma. Four explants were grown in each watch-glass. In some experiments the specimens were transferred to fresh medium every 3 days but better results were obtained when the medium was changed every 48 hours.

The watch-glass method was found to be unsuitable for the cultivation of the mandibular mesoderm owing to the very diffuse habit of growth of this tissue and its close adherence to the fibrin clot which made transference to fresh medium difficult. Large hanging-drop preparations ($1\frac{1}{4}$ inch square coverslips, $3'' \times 1\frac{1}{2}''$ hollow-ground slides) were therefore used. The explants were grown in equal parts of plasma and embryo extract. In the earlier experiments the tissue was subcultivated every three days but better results were obtained in the later experiments by transplanting to fresh medium every 48 hours. During subcultivation the central region of the explant was carefully kept intact and not subdivided. Two series of cultures were prepared in which neutral red was present in the medium in the proportion of 1 in 50,000 and 1 in 100,000 respectively.

Histological technique.

Some specimens of the normal jaw and some of the cultures of osteogenic mesoderm were fixed in neutral formalin, treated by von Kossa's silver nitrate method for the demonstration of calcification, and examined whole after being cleared in cedar wood oil or (in the case of the cultures) mounted in Farrant's medium. Many normal specimens and explants were serially sectioned. Some of the osteogenic mesoderm cultures were fixed in Flemming's solution without

acetic acid, but the rest of the material, both normal and explanted, was fixed in Bouin's or Zenker's solution. Sections were stained with Mallory's triple stain or with safranine and picro-indigocarmine.

All the explants of osteogenic mesoderm which were sectioned were cut in a plane at right angles to that of the coverglass. The explants were not removed from the coverglass on which they had been growing until they had been cleared and infiltrated with paraffin wax. The coverslips were then removed from the paraffin and the adherent wax was allowed to set, but not completely to harden. The explant, infiltrated and covered with wax, was readily peeled off the glass with a sharp knife after which it was dropped back into hot molten wax and embedded and cooled in the usual way. Tissues removed from the coverslip in this manner showed comparatively little distortion, tearing or folding.

Chemical technique.

In the previous investigation of the development of phosphatase activity in relation to the histological differentiation of embryonic limb-buds and femora grown *in vitro* and *in vivo* it was realised that the ratio of phosphatase activity to the dry weight of the tissue (A/W) was of even greater significance than the activity (A) itself. The technique employed was as follows: the phosphatase activity was first determined by measuring the amount of glycerophosphoric ester hydrolysed in 24 hours at 37° and p_H 8·6, after which the tissues were removed from the solution of glycerophosphate, rinsed in distilled water, allowed to dry in air and weighed on a micro-balance. Determination of the dry weight after measurement of the phosphatase involved a certain error, since the tissues during immersion in the glycerophosphate solution underwent some autolysis with consequent loss of weight, which was relatively greater in the early embryonic tissues than in those of later development. It has been shown [Martland and Robison, 1929] that an aqueous extract of bone phosphatase can be evaporated to dryness in an evacuated desiccator and the dry residue stored for many weeks without any loss of activity. It therefore seemed possible that the small embryonic tissue fragments and explants might be dried and weighed before estimating the phosphatase without affecting either the phosphatase content or the accuracy of its estimation. Comparative tests were carried out by both methods on corresponding bone rudiments from the right and left limbs of avian embryos at different stages of development. The values of A found by the two methods were in satisfactory agreement, and the second method was accordingly adopted for the present study. The dry weights of the tissues so determined were always greater, and the values of A/W consequently smaller, than those found by the previous method.

Details of the technique employed are as follows. The embryonic tissue or explant was placed for a moment on a piece of hardened filter paper to remove surface fluid, then transferred to a coverslip and dried for a few hours in an evacuated desiccator. The coverslips with the adherent tissues were packed in

glass tubes and transferred from Cambridge to London. They were weighed on a micro-balance after being left in the balance case until the weight remained constant. The tissues were then removed from the coverslip with a sharp knife and transferred to a small stoppered tube containing 2 cc. of water, together with a very small drop of chloroform. By reweighing the coverslips the air-dry weight (W) of the tissue was found. The tissues were kept in the chloroform water at room temperature for 3 days; 1 cc. of a $0{\cdot}2M$ solution of sodium glycerophosphate (p_H 8·7) was then added, together with another small drop of chloroform. The further procedure for the estimation of phosphatase and the method of calculating the value of A were the same as described in the previous paper [Fell and Robison, 1929, p. 769].

It should be noted that, although the value of A is quoted in the tables to 3 places of decimals, a value of 0·001 corresponding with an estimated increase in inorganic phosphate of only 0·0005 mg.P. is almost beyond the limit of experimental certainty. For this reason the more important value A/W is given only to 2 places of decimals, and when shown as less than 0·01 may in some cases have been zero.

PART I. HISTOGENESIS AND PHOSPHATASE ACTIVITY OF THE NORMAL MANDIBULAR SKELETON.

Histogenesis.

As previously stated, the mandibular skeleton of a normal late embryo (Plate VII, fig. 1) consists of Meckel's cartilage, surrounded by a sheath composed of four membrane bones—the angulare, supra-angulare, operculare and dentale [Lillie, 1919, p. 433]. Meckel's cartilage itself is not ossified except at the proximal end where it gives rise to a typical cartilage bone—the articulare.

The histogenesis of the cartilage and bone of the lower jaw was investigated in a series of 28 normal embryos ranging in age from the 6th day of incubation up to the time of hatching.

In a well developed 6-day embryo, Meckel's cartilage which is already very distinct (Plate VII, fig. 2) consists of small, rather irregular cells scattered in fairly abundant matrix and is covered by a cellular perichondrium; at a short distance from each end of the cartilage rod a zone of rather flattened cells is seen (Plate VII, fig. 2). The rod increases in size partly by apposition and partly by the formation of additional intercellular material.

After the 8th day of development the perichondrium surrounding the non-ossifying distal part of Meckel's cartilage becomes less conspicuous, the outer region growing rather diffuse and indistinguishable from the surrounding connective tissue whilst the inner cells give rise to an extremely thin fibrous membrane. The development of this part of the perichondrium is therefore different from that of the perichondrium investing an ossifying cartilage such as the femur or quadrate, which differentiates into a periosteum composed of an outer fibrous and inner osteoblastic layer.

The ossification centre of the articulare does not appear until about the 14th day of incubation but is usually fairly distinct by the 15th day. At this stage of development the ossifying cartilage shows a sharp demarcation into a middle region of large rounded, vacuolated cells, on either side of which is a broad belt of flattened cells passing into a region of small, round chondroblasts. The area of hypertrophic cells is covered by a two-layered periosteum which has formed a sheet of osteoid tissue on the surface of the cartilage, and the general histological structure of the ossifying articular cartilage closely resembles that of an ossifying long-bone rudiment from one of the limbs [Fell, 1924]. The long rod composing the distal part of Meckel's cartilage (Plate VII, fig. 1) contains somewhat flattened but irregularly shaped chondroblasts which are larger than the cells of the unossified parts of the proximal end, but are smaller than and differ markedly in appearance from the swollen, vacuolated cells of the ossifying portion.

In a 2-day post-embryonic mandible the histological structure of Meckel's cartilage is essentially the same as in a 15-day specimen. The ossification of the articulare is much more advanced, but the elongated distal part of the cartilage shows no sign of cell hypertrophy or periosteal ossification.

The formation of membrane bone begins near the articulation of the jaw. In a well developed 6-day embryo osteoblasts and a few fine osteogenic fibres can usually be distinguished in this region, but elsewhere the sites of the future membrane bones are indicated by rather diffuse condensations in the connective tissue. From the 6th to the 9th day ossification advances very rapidly and by the end of this period osteoid tissue is present as far as the apex of the jaw. Calcification begins near the articulation at about the 8th day and calcified bone is well developed throughout the mandible by the 15th day of incubation (Plate VII, fig. 1).

The histogenesis of the bone is briefly as follows.

The cells composing a condensed area of connective tissue, representing one of the four rudimentary membrane bones, are at first similar to those of the surrounding tissue. Very shortly, however, the cells in the centre of the mass enlarge somewhat and their cytoplasm becomes more deeply staining. These cells are the osteoblasts and a delicate intercellular network of osteogenic fibres rapidly forms amongst them.

The osteogenic fibres increase in number and give rise to rather irregular lamellae. Blood vessels then grow into the ossification centre and the osteoid tissue assumes an irregular trabecular structure. The intercellular material increases in density, its fibrous structure being less obvious than in the earlier stage, and at the same time many osteoblasts become enclosed in the matrix as embryonic bone cells.

The process of ossification, which is always more advanced in the original centre of ossification, extends outwards until the whole rudiment of the membrane bone is involved.

During the subsequent stages of development the intercellular material

becomes more and more plentiful whilst at the same time the bone undergoes a continual resorption and redeposition resulting at about the 15th day of incubation in the formation of a large central marrow cavity running parallel with the length of the jaw from which radiate fairly stout trabeculae between the medullary spaces.

In the 2-day post-embryonic chick these trabeculae are very thick and regular and the bone cells are scattered rather sparsely in the matrix. The marrow spaces are also larger and better defined.

Phosphatase.

The phosphatase activity of Meckel's cartilage and of membrane bone from normal embryonic and post-embryonic mandibles at different stages of development is shown in Table I. The specimens of membrane bone did not in

Table I. *Phosphatase activity of Meckel's cartilage and membrane bone from mandibles of fowl embryos and of young chicks.*

Age of embryo (days)	No. of specimens ($\frac{1}{2}$-mandibles)	Meckel's cartilage			Membrane bone		
		W mg.	*A* mg. P	*A/W*	*W* mg.	*A* mg. P	*A/W*
6	6	—	—	—	1·20	0·032	0·03
6	6	—	—	—	1·06	0·024	0·02
6	8	0·12	0·001	<0·01	—	—	—
7	6	0·27	0	0	1·38	0·042	0·03
8	2	0·09	0	0	0·52	0·007	0·01
9	2	0·15	0	0	0·57	0·035	0·06
9	2	0·20	0	0	0·75	0·055	0·07
10	2	0·20	0·001	<0·01	1·10	0·146	0·13
11	2	0·25	0·001	<0·01	1·54	0·274	0·18
11	1	0·18	0	0	1·03	0·198	0·19
13	1	0·25	0	0	2·08	0·474	0·23
15	1	0·35	0	0	2·87	0·546	0·29
Age of chick (days)							
13	1	0·62	0	0	9·04	0·68	0·08
13	1	0·60	0	0	12·77	0·92	0·07
13	1	0·94	0·011	0·01	14·20	0·74	0·05

all cases represent the entire bone of the half jaw, and the articular end of Meckel's cartilage was always rejected. It has been shown in the preceding section that during its embryonic and early post-embryonic development the distal part of Meckel's cartilage shows no sign of cell hypertrophy and does not ossify. It is seen from the table that this cartilage does not synthesise phosphatase. Specimens were examined from embryos of ages ranging from 6 to 15 days and also from 13-day chicks. In the majority of these no trace of phosphatase activity was detected and in no case did the value of A/W exceed 0·01.

Phosphatase tests on the mandibular mesoderm or membrane bone from the same embryos gave very different results, confirming the conclusions drawn from tests on human embryonic parietals [Martland and Robison, 1924] that

membrane bone as well as cartilage bone synthesises phosphatase. The 6-day and 7-day embryonic mandibles showed small but definite phosphatase activity corresponding with the presence of osteoblasts even at this early stage (*vide* p. 1909). Exceptionally, the activity of the 8-day specimen was somewhat lower, but at 9 days there was a marked increase in the value of A/W which corresponded with the rapid development of osteoid tissue noted in the preceding section. From this stage A/W rose rapidly to the high value of 0·29 in the 15-day embryos. By the 13th day of post-embryonic life the value of A/W had fallen very considerably, which can readily be accounted for by the increased proportion of inactive material (calcium salts and marrow) then present.

The maximum value of A/W found for normal embryonic femora [Fell and Robison, 1929, p. 778] was reached at the 12th day and was only 0·13 (based on the weight before extraction). The femur and another cartilage bone, the palato-quadrate, which articulates with the lower jaw, provided convenient controls in the study of the development *in vitro* of Meckel's cartilage (see next section). For this reason the phosphatase activity of normal embryonic palato-quadrates was investigated and the results are shown in Table II. The value of A/W was only 0·01 at 7 days but had risen to 0·18 in one of the 13-day specimens.

Table II. *Phosphatase development in normal embryonic palato-quadrates.*

		Palato-quadrates		
Age of embryo (days)	No. of specimens	W mg.	A mg. P	A/W
7	3	0·40	0·004	0·01
12	1	0·47	0·031	0·07
13	1	0·92	0·128	0·14
13	1	0·94	0·169	0·18
17	1	2·70	0·334	0·12

PART II. THE GROWTH, DEVELOPMENT AND PHOSPHATASE ACTIVITY OF MANDIBULAR SKELETAL TISSUE DURING CULTIVATION *IN VITRO*.

A. Meckel's cartilage.

It seemed possible that the difference in histological structure and phosphatase activity between the non-ossifying portion of Meckel's cartilage and the cartilaginous rudiment of so-called cartilage bones such as the palato-quadrate and femur might be due, not to any inherent difference in the two types of cartilage, but to some unknown factor in their respective environmental conditions. For this reason it was decided to investigate the development of Meckel's rod when removed from its normal environment and cultivated *in vitro*.

Growth and histological development.

Meckel's cartilages isolated from 6-day embryos increased considerably in both length and weight during cultivation *in vitro*, although this increase was very much less than in the case of the explanted femur.

In one experiment the entire cartilage rods from both sides of the jaw were removed from a number of 6-day embryos, explanted in a series of watch-glasses and measured at 3-day intervals during 21 days' cultivation. Twenty-four rods were explanted, 8 of which became curved and distorted during subsequent growth and had to be rejected. Four rods were dried and weighed at the beginning of the experiment.

The average length of the rods at the time of explantation was 3·4 mm. and after 21 days' cultivation was 5 mm., showing an increase of 47 %. The greatest increase in length of any individual specimen was 80 %. The original average length of the explanted 6-day femora [Fell and Robison, 1929] was 2·47 mm. and during 21 days' cultivation this length increased by 118 %.

The average dry weight of the control Meckel's cartilages was 0·048 mg. and that of the explants after 21 days' cultivation was 0·091 mg. The increase during growth *in vitro* thus amounted to 90 % of the original weight.

In order to study the histological development and phosphatase activity of the non-ossifying part of the mandibular cartilage when cultivated *in vitro*, a number of 6-day embryonic Meckel's rods from which the articular end had been removed were explanted in watch-glasses. An equal number of cultures of either the palato-quadrates or the femora from the same embryos were made as controls. In most of the experiments some of the explants were fixed and sectioned after 18–20 days' cultivation and the remainder were tested for phosphatase. In one experiment explants of Meckel's cartilage and of the palato-quadrate were fixed after 2, 4, 8 and 16 days' growth *in vitro* in order to observe the histological structure at different stages of development. In all, sections were made of 36 Meckel's rods controlled by 36 palato-quadrates and of 10 Meckel's rods controlled by 9 femora.

The experiments with palato-quadrate controls were perhaps the more interesting owing to the fact that the quadrate and Meckel's cartilage are adjacent structures.

In a 6-day embryo the palato-quadrate already shows its typical tri-radiate form which was usually preserved during the first 8 days' growth *in vitro* during which time the quadrate increased considerably in size. After this period, however, the explants usually became rather deformed owing to twisting and bending of the arms. The explants of Meckel's cartilage also tended to become slightly distorted after this stage (Plate VII, fig. 3) usually by a hook-like bending of one or both ends. This distortion of the explants was probably due to the increasing toughness of the fibrous capsule by which the growing cartilage was surrounded.

There is no conspicuous histological difference between the quadrate and the Meckel's rod in the 6-day embryo (Plate VII, fig. 2). Both consist entirely of small-celled cartilage showing no trace of periosteal ossification.

After 2 days *in vitro* the structure was still essentially the same in both types of explant. Both showed an increased amount of matrix and contained

rather irregularly shaped chondroblasts, some of which showed mitotic figures. All the explants were covered by a layer of connective tissue.

After 4 days *in vitro* the Meckel's rods and some of the smaller quadrates were unchanged in microscopical appearance, but in the larger quadrates a slight swelling of the cells occupying the middle region of one of the three arms could be observed, associated with a flattening of the cells bordering this area.

In 8-day cultures a striking difference in the histological structure of the two sets of explants was seen. Sections of the six palato-quadrates fixed at this stage all showed, as in the normal development of this cartilage *in vivo*, a well marked differentiation into three cell zones, *viz.* a middle region of round, vacuolated, hypertrophic cells bounded on each side by a belt of flattened cells which in turn passed into an area of small, round chondroblasts. In some cases a delicate layer of osteoid tissue had been deposited on the surface of the hypertrophic region. The Meckel's cartilages, on the other hand, still consisted of irregularly shaped cells scattered in fairly plentiful matrix.

In the older (16–20 day) explants the same contrast between the Meckel's rods and the palato-quadrates was observed. All the quadrates (Plate VII, fig. 4) showed the three definite regions of small, flattened and hypertrophic chondroblasts characteristic of normal "cartilage-bone" rudiments, whilst all the Meckel's cartilages (Plate VII, fig. 3) contained only irregularly shaped cells similar to those seen in Meckel's cartilage of a normal late embryo (Plate VII, fig. 1). Most of the quadrates showed a certain amount of periosteal ossification. The only ossification ever observed in the explants of Meckel's cartilage were small areas of membrane bone in the surrounding tissue, which were quite unassociated with the cartilage; such areas only occurred in a few isolated cases and disappeared during the later stages of cultivation.

In those experiments where the femora instead of the quadrates were used as controls, similar results were obtained, *i.e.* well developed zones of small, flattened and hypertrophic cells were seen in all the femoral cultures but did not appear in a single explant of the jaw cartilage.

From these results it was clear that the striking contrast in histological structure between the non-ossifying part of Meckel's rod and ossifying cartilage such as the embryonic quadrate or femur cannot be due to the influence of environmental factors since each type develops its characteristic histology when isolated from the body and growing in a watch-glass.

Phosphatase.

The results of phosphatase tests on explants of the distal part of Meckel's cartilage cultivated 17–19 days *in vitro* are shown in Table III, and are there compared with the corresponding values for explants of palato-quadrates or femora from the same embryos cultivated *in vitro* for the same length of time. The results are in entire agreement with the histological differences described in the preceding section.

Table III. *Phosphatase development in cultures of Meckel's cartilages compared with that in palato-quadrates and femora of 6-day fowl embryos.*

Exp.	Duration of cultivation (days)	No. of explants	Meckel's cartilages			Palato-quadrates		
			W mg.	*A* mg. P	*A*/*W*	*W* mg.	*A* mg. P	*A*/*W*
1	0	6	0·18	0·001	<0·01	0·24	0·002	<0·01
1*	17	18	0·74	0·003	<0·01	1·00	0·099	0·10
2	0	8	0·12	0·001	<0·01	0·16	0	0
2†	19	18	1·16	0·003	<0·01	1·48	0·48	0·33
						Femora		
3	19	3	0·17	0·001	<0·01	1·22	0·154	0·13
3	19	3	0·20	0·001	<0·01	1·03	0·081	0·08
3	19	3	0·19	0·001	<0·01	1·12	0·084	0·08
3	19	3	0·25	0·001	<0·01	—	—	—
4	19	18	0·91	0	0	—	—	—
4	19	3	—	—	—	0·81	0·035	0·04
4	19	3	—	—	—	1·07	0·162	0·15
4	19	3	—	—	—	0·93	0·093	0·10
4	19	3	—	—	—	1·06	0·087	0·08
4	19	3	—	—	—	0·80	0·045	0·06
4	19	3	—	—	—	0·85	0·046	0·05

* Transplanted every 3 days. † Transplanted every 2 days.

In the first three experiments the very slight traces of phosphatase found in the explants were no greater than those found in the controls, and represented values of A/W of less than 0·01. In order to obtain a more critical test the whole of the 18 explants in Exp. 4 were tested together, giving a total air-dry weight of 0·91 mg., but no phosphatase at all was detected, in agreement with the complete absence of hypertrophic cells and ossification. On the other hand, the explants of palato-quadrates and femora in which cell hypertrophy and ossification occurred were found to have synthesised phosphatase during cultivation. It was interesting also to find that the value of A/W determined for these explants corresponded very closely with the histological condition observed in explants from the same cultures, which had been fixed and sectioned. Thus, in Exp. 1, in which the culture medium was changed every 3 days, some degeneration of the palato-quadrate explants was seen, and a relatively low value of A/W (0·10) was found. In Exp. 2 the culture medium was changed every 48 hours and very little degeneration of the palato-quadrates was observed; the value of A/W found for 3 explants was 0·33, the highest yet recorded. Similarly in the cultures of femora in Exp. 4 the rather variable values of A/W corresponded with the general superficial appearance of the explants, those showing the least abnormality giving the highest phosphatase values.

B. Membrane bone.

Histological development.

It was found that when the soft tissue surrounding Meckel's cartilage in the 6-day embryonic jaw was dissected away and explanted *in vitro*, about 65 % of the explants developed ossification centres. Ossification was observed in over 180 cultures and the various stages in the process could be seen in the living tissue.

In most of the experiments two explants were made from each half jaw and two entire jaws were fixed and sectioned to serve as controls. In some experiments cultures were made from half the jaw whilst the other half was fixed and sectioned so that each pair of cultures had a control. Twenty-three entire or half jaws were used as controls in this way.

Examination of this control material showed that in some cases there was no trace of ossification but in the better developed specimens a small area of osteoblasts with a few osteogenic fibres could be distinguished near the articulation of the jaw though elsewhere the future membrane bones, as described above, were indicated only by diffuse condensations in the connective tissue surrounding Meckel's cartilage.

When first explanted the tissue, consisting mainly of connective tissue and myoblasts, was usually rather diffuse, but after 24 hours' growth *in vitro* it had contracted into a more or less compact mass of cells of which the outermost had begun to wander into the medium forming a narrow fringe of outgrowth around the central fragment.

During the next 24 hours' cultivation the compact mass gradually spread over the coverslip in a sheet of tissue which usually showed in places one or more fairly large aggregations of cells merging imperceptibly into the surrounding tissue (Plate VIII, fig. 5 *a*). Subsequent observation showed that in many instances such a group of cells represented the first appearance of an ossification centre. In these cases the group rapidly became more distinct and more restricted and after 3 days *in vitro* was often seen as a well defined, usually oval area of somewhat closely packed cells which seemed to be separated from each other by slightly refractile and apparently amorphous intercellular substance. The cells of the ossification centre took up neutral red to about the same extent as the surrounding connective tissue cells.

Histological sections of a culture containing such a centre showed the latter to consist of an oblong mass of cells of somewhat irregular shape and rather deeply staining cytoplasm which were slightly flattened in a direction at right angles to the coverslip; the intercellular spaces contained a loose coagulum.

The next stage in development, which usually took place within the following 24 hours, was the differentiation of the cell mass into two regions—an inner part composed of polyhedral cells and an outer capsule of elongated cells continuous with the surrounding tissue. In cultures in which neutral red

was present in the medium the inner cells were conspicuous owing to the readiness with which they stained with the dye.

A few hours later (Plate VIII, fig. 5 *b* and Plate IX, fig. 8) very delicate intercellular fibres—the osteogenic fibres—appeared in the inner region of the ossification centre. These increased in number until they formed a fairly fine network between the closely packed cells. Usually the inner region now consisted of a single peripheral layer of osteoblasts surrounding a central mass of early bone cells embedded in the osteogenic fibres, whilst the outer region had given rise to a typical fibrous capsule of connective tissue. The osteoblasts and the young bone cells in the neutral red cultures stained even more deeply and examination under high power showed that the cytoplasm was filled with rather fine, spherical red granules.

This type of structure was attained by about the 4th or 5th day *in vitro* (Plate VIII, fig. 5 *c*). Sections of such a specimen (Plate IX, fig. 9) showed the fibrous capsule and adjacent layer of osteoblasts described in the case of the living cultures. The inner mass of developing bone was about 4–8 cells thick; the oblong or polyhedral bone cells were set closely and fairly regularly in the fibrous intercellular material which with Mallory's stain and with safranine and picro-indigocarmine gave exactly the same staining reactions as in the normal jaw.

The next stage observed (Plate VIII, figs. 5 *d* and 6) in developing ossification centres was the formation of an apparently amorphous cement between the osteogenic fibres. In one culture where a very thin ossification centre was present the various stages in this process could be followed particularly well in the living material. It was found that the criss-cross network of delicate fibres originally seen became aggregated into refractile bundles in which the individual fibres were difficult to distinguish. Focussing on the surface of the capsules with the high power of the microscope the matrix was seen to have a matted structure very different in appearance from the delicate network observed some 24 hours previously. The density and refraction of the matrix progressively increased whilst the fibres became less and less conspicuous, being probably obscured by the cement in which they were embedded.

As the age of the ossification centre advanced it was found that in some areas the original intercellular partitions were resorbed and replaced by small cavities filled with osteoblasts, in other places they remained apparently unaltered, whilst elsewhere they became thickened to form fairly stout, irregular walls and columns. In this way the developing bone gradually assumed an irregular trabecular structure which was usually well seen about the 9th or 10th day *in vitro*. Whilst these changes were taking place in the general structure of the matrix, a certain proportion of the osteoid nodules underwent partial and occasionally (Plate VIII, fig. 7) extensive calcification. Refractile granules appeared in the intercellular material and increased in number until the bone appeared almost black when viewed under the low power of the microscope by transmitted light. When examined under high power the bony

trabeculae were seen to have a nodular appearance owing to the brilliant granules embedded in the tissue. Such a culture fixed in neutral formalin and stained with silver nitrate showed a dense, granular deposit of silver in the bone (Plate VIII, fig. 7).

Sections of a culture at this stage of development (Plate IX, fig. 10) confirmed the observations made on the living material. A nodule of bone was seen surrounded by a capsule of very cellular connective tissue within which was a layer of osteoblasts. The bone itself contained irregular spaces filled with osteoblasts and connective tissue cells. The bone corpuscles were normal in appearance and scattered throughout the dense matrix which was hard and difficult to cut.

Besides the compact ossification centres described above, diffuse ossification centres also occurred, though less frequently. In such cases there was no definite periosteum and at the margin of the developing bone the osteogenic fibres passed into the surrounding connective tissue in all directions. A compact and a diffuse centre were sometimes present in the same culture, and in other cases the same ossification centre might be compact at one end and diffuse at the other.

The rate of development of the bone varied considerably in different cultures so that one explant might take 9 days to reach a stage of differentiation attained by another after 6 days *in vitro*.

The tissue surrounding the ossification centres was complex and consisted of connective tissue, myoblasts, wandering cells and, when embryos of coloured breeds were used, branched pigment cells.

In some cases (Plate IX, fig. 11) the bone remained in a fairly healthy condition up to the 14th and more rarely up to the 24th day of cultivation. Usually, however, the ossification centres began to disappear after about the 12th day *in vitro* and eventually vanished. In this respect the membrane bone cultures differed from the cultures of periosteal bone described in a previous communication [Fell, 1928], as in some instances the latter contained healthy bone and osteoid tissue after 84 days' cultivation. This point is being investigated further.

Phosphatase.

The results of phosphatase tests on the explants of mandibular mesoderm grown *in vitro* for periods of 6 to 8 days are shown in Table IV. Ossification centres had developed in the majority of these explants during cultivation and an increase in the phosphatase activity of the tissues was therefore expected. In all these experiments a considerable increase in the value of A/W was actually observed for the explants as compared with that for the controls. The maximum increase was found in Exp. 2, in which the value of A/W for explants after 6 days' growth *in vitro* was 0·13. It will be seen that in Exp. 1 a higher value was obtained for those explants which contained ossification centres than for those in which no such centres had developed.

Table IV. *Phosphatase development in membrane bone grown in vitro from the mandibular mesoderm of 6-day embryos.*

(Four explants made from one mandible.)

Exp.	Duration of cultivation (days)	No. of specimens	*W* mg.	*A* mg. P	*A*/*W*	Remarks
1	0	1 mandible	0·31	0·003	0·01	
	6	14 explants	0·23	0·016	0·07	All contained ossification centres
	6	7 explants	0·22	0·005	0·02	Contained no ossification centres
2	0	3 mandibles	1·06	0·024	0·02	
	6	12 explants	0·27	0·036	0·13	
3	0	3 mandibles	1·20	0·032	0·03	
	8	15 explants	0·30	0·033	0·11	

The proof that phosphatase was synthesised during cultivation of the explants *in vitro* is not, however, so clear as in the experiments with femora and palato-quadrates, since in spite of the marked increase in the ratio A/W the total phosphatase activity (A) of the explants after cultivation was, in two of the experiments (Exps. 1 and 3), actually less than that of the controls (bearing in mind that four explants were made from one mandible). This was due to the fact that the weight of the explants was much smaller than that of the controls. The difference in the weight resulted from two causes; in the first place it was necessary to dissect off the ectoderm from the tissue which was to be explanted and this somewhat difficult operation involved the removal of a considerable quantity of the underlying mesoderm; in the second place, during each subcultivation, only the central part of the tissue was transplanted, a considerable portion of the outgrowth being rejected. If the rejected tissue were entirely devoid of phosphatase the increase in the ratio A/W could be accounted for without assuming any synthesis of the enzyme. In Exp. 2, however, although the weight of the 12 explants was only one quarter of the weight of the 3 mandibles used as controls, the total phosphatase activity (A) was increased by 50 %. It is therefore very probable that synthesis of phosphatase occurred during development of this membrane bone *in vitro* as it certainly does during the normal growth of the bone *in vivo*.

Discussion.

The results recorded in this communication indicate that by the 6th day of embryonic development the future histological structure of the non-ossifying part of Meckel's rod and of a future cartilage bone such as the palato-quadrate or femur is already firmly determined, although at this stage no histological differences between the two types of cartilage have yet appeared. It has been shown [Strangeways and Fell, 1926; Fell, 1928] that at an earlier stage of development the histological destiny of the chondrogenic tissue of the limb-rudiment is not so definitely determined since the undifferentiated 3-day limb-bud when cultivated *in vitro* usually gives rise to nodules of small-celled, non-

ossifying cartilage. On the other hand, Murray and Selby [1930] state that the early (2-day) embryonic limb-rudiment, when removed from the body and grafted on to the chorio-allantoic membranes, may form "ossifying" cartilage with typical hypertrophic cells, showing clearly that the isolated rudiment, even at this early stage, has the potentialities for its characteristic histological differentiation when growing under suitable environmental conditions. The histological similarity between the non-ossifying part of Meckel's rod and the non-ossifying cartilage formed in the explanted 3-day limb-bud does not necessarily mean that the two types of cartilage are identical, and it is probable that the same histological result is differently produced in the two cases. The factors responsible for determining whether chondrogenic tissue shall develop into non-ossifying cartilage or into ossifying cartilage with the characteristic hypertrophic cells are at present quite obscure.

The close correlation which exists between the histological structure and the occurrence of phosphatase in cartilage has again emerged from this study of the palato-quadrate and of Meckel's rod. In the normal embryonic palato-quadrate, as in the femur, the progressive development of hypertrophic cartilage cells and of osteoid tissue was accompanied by rapidly increasing phosphatase activity, while the non-ossifying cartilage from Meckel's rod remained inactive even in early post-embryonic life.

The specific biochemical character associated with these different types of cartilage was equally manifested when the 6-day embryonic rudiments were removed from the body and allowed to develop *in vitro*. At the time of explantation both the palato-quadrates and Meckel's cartilage were almost devoid of phosphatase, but during cultivation, marked synthesis of the enzyme occurred in the palato-quadrates along with chondroblastic hypertrophy and ossification, whilst in the Meckel's cartilage neither hypertrophic chondroblasts, periosteal ossification, nor phosphatase were found.

The fact that synthesis of phosphatase also accompanies ossification in membrane bone found similar confirmation from the cultivation of such bone *in vitro*. Although the evidence for the synthesis of phosphatase *in vitro* was less clear than that obtained in the cultivation of femora and palato-quadrates, the appearance of ossification centres in explants of mandibular mesoderm was accompanied by a large increase in the ratio of phosphatase to the dry weight of tissue.

Summary.

1. The histological development of the normal embryonic mandibular skeleton, consisting of Meckel's cartilage surrounded by a sheath of membrane bone, was investigated.

2. The non-ossifying distal part of Meckel's rod in a late embryo differs from the cartilage of the palato-quadrate or femur in the absence of the region of vacuolated, hypertrophic cells characteristic of ossifying cartilage.

3. The correlation between the histological structure of cartilage and

phosphatase synthesis is made evident in these three cartilage rudiments. The palato-quadrate and femur develop high phosphatase activity while the non-ossifying part of Meckel's rod remains devoid of phosphatase up to an early stage of post-embryonic life.

4. The membrane bone surrounding Meckel's cartilage possesses a high phosphatase activity.

5. The distal part of Meckel's rod was removed from a number of 6-day embryos and explanted *in vitro* by the watch-glass method. Similar cultures were made of either the palato-quadrates or femora from the same embryos. As in the normal embryo, the distal part of Meckel's cartilage when cultivated *in vitro* neither ossified nor formed a region of hypertrophic chondroblasts, whilst in the explanted palato-quadrates and femora an area of hypertrophic cells appeared which was usually associated with a layer of osteoid tissue.

6. During cultivation *in vitro* the explants of Meckel's cartilage synthesised no phosphatase while the explanted palato-quadrates and femora developed high phosphatase activity.

7. The mandibular mesoderm surrounding Meckel's cartilage was removed from a large number of 6-day embryos and explanted *in vitro* by the hanging-drop method. Ossification centres developed in about 65 % of the cultures. It was possible to observe the different stages of osteogenesis in the living material.

8. There was evidence that explants of mandibular mesoderm in which ossification took place also synthesised phosphatase.

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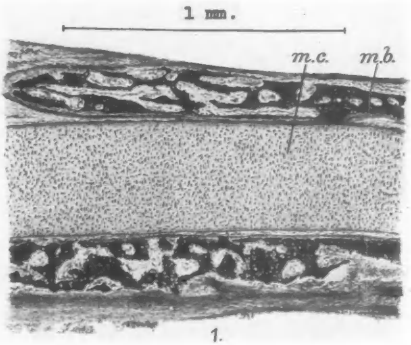

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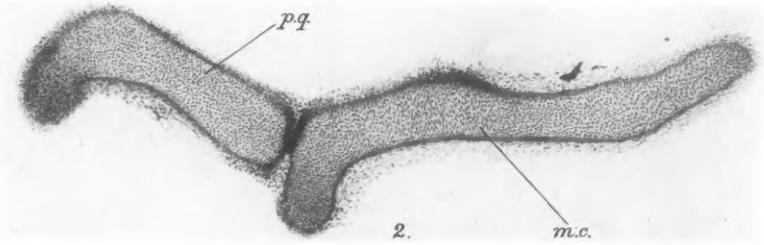

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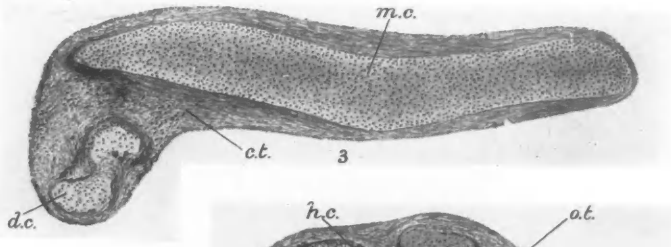

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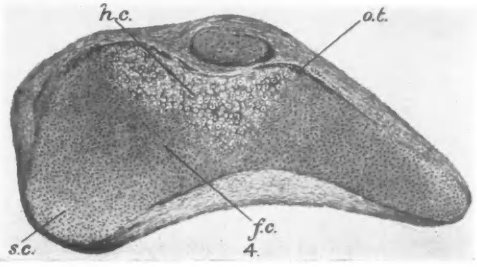

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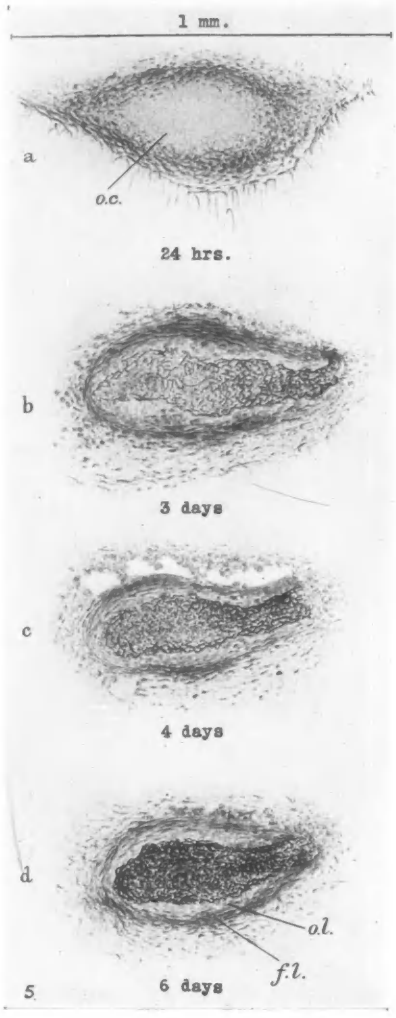

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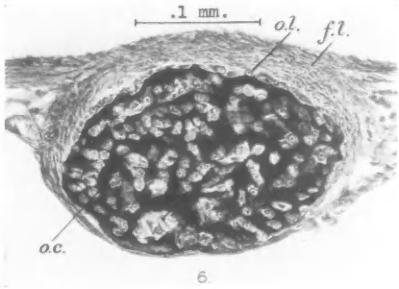

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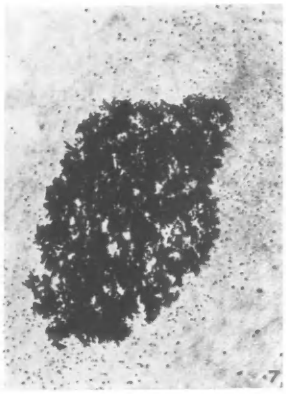

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DESCRIPTION OF PLATES.

c., cartilage; *c.t.*, connective tissue; *d.c.*, degenerate cartilage; *f.c.*, flattened chondroblasts; *f.l.*, fibrous layer; *h.c.*, hypertrophic chondroblasts; *m.b.*, membrane bone; *m.c.*, Meckel's cartilage; *p.q.*, palato-quadrate; *o.c.*, ossification centre; *o.l.*, osteoblastic layer; *o.t.*, osteoid tissue; *s.c.*, small chondroblasts.

PLATE VII.

Fig. 1. Longitudinal section through the normal mandibular skeleton of a 15-day fowl embryo. Note the rod of unossified cartilage and the early membrane bone.

Fig. 2. Longitudinal section through the normal palato-quadrate and Meckel's cartilage of a 6-day fowl embryo (control specimen).
The three regions of small, flattened and hypertrophic cells have not yet appeared in the palato-quadrate, the histological structure of which is almost indistinguishable from that of the adjacent Meckel's cartilage.

Fig. 3. Longitudinal section of an explant of the distal part of Meckel's cartilage from a 6-day embryonic jaw after 20 days' cultivation *in vitro*.
This explant was obtained from the same set of embryos as the control shown in Fig. 2. There is no differentiation into the three zones of small, flattened and vacuolated, hypertrophic cells seen in normal ossifying cartilage and the histological structure of the explanted Meckel's rod resembles that of the normal specimen shown in Fig. 1.

Fig. 4. Section through an explant of the palato-quadrate from a 6-day embryo after 20 days' cultivation in a watch-glass.
The explant was taken from the same set of embryos as the two preceding specimens. Note the three zones of cartilage cells (small, flattened and hypertrophic) which have developed during cultivation although (*cf.* control) these regions were not differentiated at the time of explantation. This specimen also shows slight periosteal ossification over the area of hypertrophic cells.

PLATE VIII.

Fig. 5. Osteogenesis in a hanging-drop culture of mesoderm from a 6-day embryonic jaw. The figures are drawn from camera lucida sketches of a living culture and were made at different stages of growth to show the development of an ossification centre. After 6 days' cultivation this specimen was fixed and sectioned. A section is shown in Fig. 6.

Fig. 6. Histological section of culture shown in Fig. 5 after 6 days' growth *in vitro*.

Fig. 7. Photograph of an 8-day coverslip culture of the soft tissue from a 6-day embryonic jaw. This specimen was stained with silver nitrate and mounted whole. An ossification centre, which developed during cultivation, stained black with the silver nitrate indicating that true calcified bone had been formed.

PLATE IX.

Fig. 8. Section of a similar culture after 2 days' growth *in vitro*.
Note the two very early ossification centres which appeared during cultivation. Only a few osteogenic fibres are present at this stage.

Fig. 9. Section of a similar culture after 6 days' growth showing an ossification centre which developed *in vitro*. The osteoid tissue has reached a higher stage of differentiation than that represented in Fig. 8.

Fig. 10. Section of a similar culture after 6 days' cultivation. Two ossification centres and a smal nodule of cartilage are shown (the presence of cartilage is exceptional).

Fig. 11. Section of a similar culture after 14 days' growth *in vitro*. Note the mass of trabecular bone which has differentiated during cultivation.

CCXVI. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

VIII. CALCIFICATION *IN VITRO*.

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In the first two papers of this series [Robison, 1923; Robison and Soames, 1924] it was shown that when bones of rachitic rats were immersed in solutions of calcium hexosemonophosphate or glycerophosphate, a dense deposit of calcium phosphate was formed in the matrix of the hypertrophic cartilage, where calcification would have occurred *in vivo* if the rats had been fed on a normal diet. The location of the bone phosphatase and its power to effect calcification were thus demonstrated, since only by the agency of this enzyme could the insoluble calcium phosphate have been produced from the phosphoric ester. It was further suggested that a second mechanism might also play a part in normal calcification by bringing about a slight increase in the p_H of the matrix fluid, thereby increasing the concentration of PO_4 and CO_3 ions, and also the activity of the enzyme.

Shipley [1924] found that slices of cartilage and bone of a rachitic rat became calcified when placed in the serum of normal rats and kept at 37° for a number of hours, and later it was shown by Shipley, Kramer and Howland [1926] that calcification could be effected by immersing such bone slices in solutions of inorganic salts, provided that "the concentrations of the bone-forming constituents and the reactions are nearly those of normal serum." In a reply [Robison, 1926] to the criticism of the enzyme hypothesis by these authors it was pointed out that the apparent similarity of the concentrations of calcium and inorganic phosphate in their calcifying solutions with those in normal serum was misleading, owing to the absence from these solutions of proteins and other organic constituents, which greatly depress the ionisation of calcium in plasma. These solutions were, indeed, highly supersaturated with respect to the bone salt, but the fact that calcification, normal in character, occurs when such solutions are allowed to diffuse into the cartilage is none the less of great significance.

Our own experiments[1] confirm the results of Shipley, Kramer and Howland

[1] The results described in this paper were communicated to the Biochemical Society in Feb 1928 [1928].

in nearly all respects. These authors found that calcification was obtained with salt solutions for which the value of [Ca] × [P] was 35, while in the experiments described in the present paper calcification did not occur with values of [Ca] × [P] below 40 and only rarely at this level. This apparent divergence in our results is probably accounted for by the great difference in the concentrations of sodium chloride in the solutions used. According to a footnote in a later paper by Shipley and Holt [1927] the concentration of sodium chloride in the solutions used by Shipley, Kramer and Howland was only 0·013 *M*, and not 0·1 *M* as stated in the original description of their experiments. The later work of Shipley and of Kramer and their collaborators and the experiments described in Part IX of this series [Robison, Macleod and Rosenheim, 1930] show that the level of the product [Ca] × [P] required for calcification depends to a considerable extent on the concentrations of sodium chloride and of the other salts present in the solution.

The experiments described in the present paper deal with the effect of phosphoric ester on calcification *in vitro* in solutions containing calcium and inorganic phosphate in low concentrations. They prove that while calcification does undoubtedly take place in absence of phosphoric ester, provided the value of [Ca] × [P] is sufficiently high, a much lower level of this product is required when phosphoric ester is present even in very small amounts.

Method of experiment.

For these experiments we used bones of young rats and of rabbits which had been fed for three to four weeks on a rickets-producing diet (McCollum 4026, 3143 or Steenbock 2965). The animals were killed by a blow on the neck and the bones were removed with precautions to avoid bacterial contamination. The distal heads of the femora and the proximal heads of the tibiae were used, the latter being preferred on account of the broad regular zone of epiphysial cartilage. The head of the bone was either cut in half longitudinally (as in our former experiments), one half being placed in each solution, or into a number of thinner longitudinal slices. For this purpose a sterile safety razor blade was found convenient. One half-head or slice was stained with silver nitrate and examined for calcification due to healing. Experiments in which such healing was observed in the controls are not included in the table.

The basal solution used in most of these experiments had the following composition:

	g. per 100 cc.	*mM*
NaCl	0·6	103
$NaHCO_3$	0·03	3·6
KCl	0·04	5·4
$MgSO_4$, $7H_2O$	0·025	1·0
Phenol red	0·0003	—

In the experiment shown in Table I the solution contained 0·9 % NaCl and in Exp. 1 and 2 of Table II the solutions contained 0·7 % NaCl, but were otherwise similar to the above.

A stock solution was made up of twice this salt concentration and diluted after addition of calcium chloride, potassium phosphate and glycerophosphoric ester as required to give the concentrations of calcium, inorganic P and organic P stated in the tables. The methods of preparation and sterilisation of these solutions and the adjustment of p_H are described in the next paper [Robison, Macleod and Rosenheim, 1930]. The half-heads or bone slices were immersed in the solutions, previously warmed to 38°, for the stated periods and were then removed, fixed in 10 % neutral formalin, washed, and stained by von Kossa's silver nitrate method. The extent of new deposit, if present, was judged by examining the slice under low magnification and is shown in the Tables by a numeral, the maximum value, 10, indicating that the deposit extended over the entire area of hypertrophic cartilage. In the later experiments the slices were dehydrated and cleared before examination.

Results.

Table I shows the results of an experiment in which slices from the femora and tibiae of the same rachitic rat were immersed in solutions containing constant concentrations of calcium and of inorganic phosphate but different concentrations of phosphoric ester. Each slice was placed in a small stoppered flask, containing 40 cc. of the sterile solution at p_H 7·35, and kept at 38° during 16 hours. The extent to which calcification occurred *in vitro* is approximately indicated by the number in the last column, and is shown in the photomicrographs reproduced in Figs. 1–6 (Plate X).

Table I.

Solution	mg. per 100 cc. Ca	Inorg. P	Org. P	Relative extent of new calcification (max. 10)
A	10	3·5	0	0
B	10	3·5	0·5	1
C	10	3·5	1	4
D	10	3·5	2	6
E	10	3·5	4	8

No calcification occurred in any of the slices immersed in solution *A*, which contained no phosphoric ester. The addition of glycerophosphate equivalent to only 0·5 mg. organic P per 100 cc. caused some calcification to occur, while the extent of the deposit was increased with each further increase in the amount of ester.

Apart from the store of hydrolysable phosphoric esters in the red corpuscles such esters have been shown to occur in plasma in amounts usually between 0·2 and 0·5 mg. P per 100 cc. but sometimes exceeding this limit [Martland and Robison, 1926].

In drawing conclusions from the results of this and similar experiments, objection might be raised to one point. During the immersion of the bone slices in the solution containing phosphoric ester, a slight rise occurred in the concentration of inorganic phosphate in the external solution, due partly to

diffusion of enzyme from the bone and partly to diffusion of some of the phosphate set free by enzyme hydrolysis within the cartilage. The rise did not usually exceed 0·5 mg. P per 100 cc. and occurred chiefly towards the end of the experiment. It was, moreover, due directly to the phosphatase activity. It was desirable, nevertheless, to avoid confusing the argument by these variations in the composition of the surrounding fluid. We therefore carried out a number of experiments in which the sterile solutions were caused to pass in a slow continuous stream over the bones in the apparatus shown in Fig. 1.

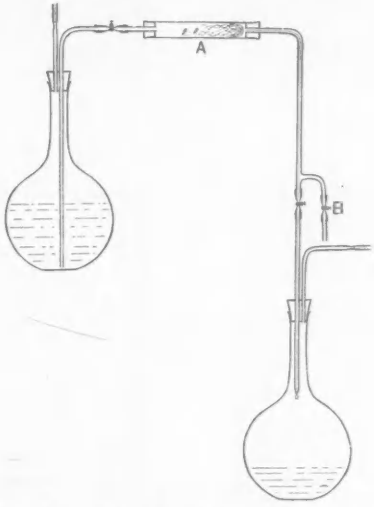

Fig. 1. *A*, tube containing bone slices and glass wool plug; *B*, outlet for withdrawal of samples of the solution.

Two similar sets of apparatus were used, each containing 2 litres of solution which was allowed to flow over the bones during 20 to 48 hours, the temperature being maintained at 38°. Tests carried out on samples of the solution which had passed over the bones showed that the concentration of inorganic phosphate had not risen appreciably but that phosphatase continued to diffuse out of the bones at an increasing rate throughout the whole period. The total phosphatase activity of the solution in the lower flask was so great as to suggest that the living cartilage cells were continuing to produce and secrete the enzyme, but further experiments did not confirm this. The continuous loss of enzyme must involve a severe handicap to the phosphatase mechanism. Further, the concentration of inorganic phosphate was always made higher in the inorganic solution than in the solution containing phosphoric ester. Nevertheless, the results given in Table II show that calcification is regularly obtained in presence of ester at levels of calcium and inorganic phosphate which produce no calcification when ester is absent.

Table II.

The concentrations of Ca, inorganic P and organic P are given in mg. per 100 cc. The inorganic solution contained no phosphoric ester. The letters *h* and *s* in column 3 indicate half-heads of bones, and slices, respectively. Where more than one value is shown for calcification these refer to the separate bone slices immersed in the same solution.

					Inorganic solution			Organic solution			
Exp.	Animal		p_H	Duration hrs.	Ca	Inorg. P	Calcification (max. 10)	Ca	Inorg. P	Org. P	Calcification (max. 10)
1	Rat	*h*	7·2	22	10	4	0	10	3·8	3	5
2	,,	*h*	7·4	18	10	4	0	10	3·5	6	10
38	,,	*s*	7·35	28	10	4	0, 0	10	3·5	3	5, 5
41	,,	*s*	7·3	24	10	3·6	0, 0	10	3·2	3	8, 9
		h	7·3	24	10	3·6	0	10	3·2	3	8
46	,,	*s*	7·3	24	—	—	—	10	2·8	3	3
								10	2·8	9	9
24	Rabbit	*s*	7·3	22	10	5·5	10, 10, 10	10	5	4	10, 10, 10
7	,,	*s*	7·4	18	10	4	2, 2, 1, 0	10	3·5	3	4, 3, 3, 3
3	,,	*s*	7·35	18	10	4	0, 0, 0	10	3·5	3	10, 3, 2
13	,,	*s*	7·3	24	10	3·75	0, 0, 0, 0	10	3·5	3	10, 10, 10, 10
4	,,	*s*	7·3	18	10	3·5	0, 0, 0	10	3	3	4, 0
26	,,	*s*	7·4	18	7·5	4·5	0, 0, 0, 0	7·5	4	9	6, 5, 3, 2
8	,,	*s*	7·5	27	7·5	4	0, 0, 0	7·5	3·5	3	4, 0, 0
14	,,	*s*	7·3	48	7·5	3·75	0, 0, 0	7·5	3·5	3	7, 3, 1
15	,,	*h*	7·3	48	7·5	3·75	0	7·5	3·5	1	2
21	,,	*s*	7·4	24	5	5	0, 0	5	4·5	3	2, ½
18	,,	*h*	7·2	24	5	5	0	5	4	3	1

Summary.

It is shown that while calcification *in vitro* may occur in solutions of inorganic salts supersaturated with respect to the bone salt, calcification will take place with lower concentrations of calcium and inorganic phosphate if phosphoric ester is also present, even in very small amount.

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DESCRIPTION OF FIGURES IN PLATE X.

Figs. 1–6. Slices cut from the distal heads of the femora and proximal heads of the tibiae of a rachitic rat. The slices were immersed 16 hours at 37° in solutions all containing 10 mg. Ca and 3·5 mg. P as inorganic phosphate per 100 cc., but containing different concentrations of glycerophosphoric ester. Slices stained by von Kossa's silver nitrate method and photographed, without clearing, by reflected light. Mag. × 11.
X = new deposit of calcium salts in the zone of hypertrophic cartilage (see Table I).

Fig. 1.	Femur slice in solution	*A*,	Phosphoric ester	≡0		
Fig. 2.	,, ,, ,,	*B*,	,,	≡0·5 mg. P per 100 cc.		
Fig. 3.	,, ,, ,,	*C*,	,,	≡1	,,	
Fig. 4.	Tibia ,, ,,	*A*,	,,	≡0		
Fig. 5.	,, ,, ,,	*D*,	,,	≡2	,,	
Fig. 6.	,, ,, ,,	*E*,	,,	≡4	,,	

Fig. 1.

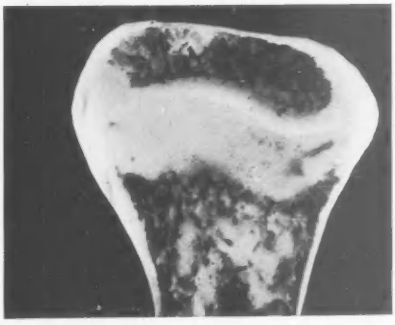

Fig. 4.

Fig. 2.

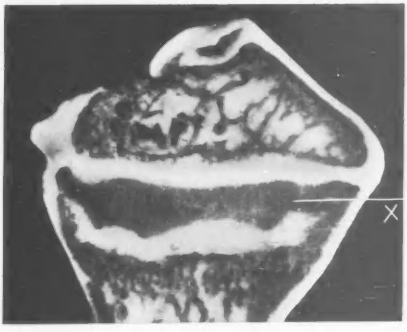

Fig. 5.

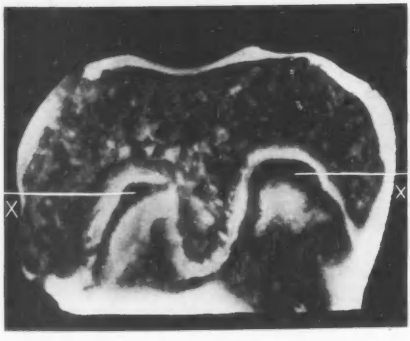

Fig. 3.

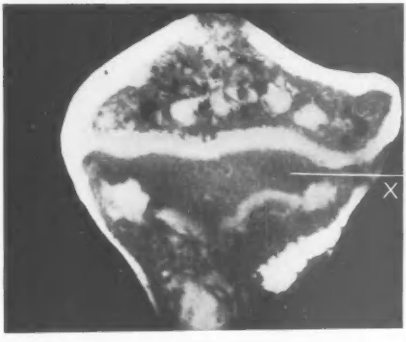

Fig. 6.

CCXVII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

IX. CALCIFICATION *IN VITRO*.

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In the preceding paper [Robison and Soames, 1930] it was shown that, although bone slices from rachitic animals will calcify in solutions of inorganic salts supersaturated with respect to the bone phosphate, at lower concentrations of calcium and inorganic phosphate calcification will only occur in presence of phosphoric ester. At critical concentrations of the inorganic radical as little as 0·5 mg. per 100 cc. of phosphorus in the form of ester is sufficient to determine calcification, this amount being similar to that found in plasma. The solutions used for these experiments contained no protein and no other organic constituent but phosphoric ester. The concentration of calcium ions was, therefore, higher than in plasma even when the total calcium content was the same. The solutions further differed from plasma in containing much less bicarbonate.

The experiments described in the present paper deal with the effect of bicarbonate, sodium, potassium and magnesium on calcification in protein-free solutions at different levels of calcium and inorganic phosphate, in absence and in presence of phosphoric ester. Experiments on the effect of antiseptics, of previous treatment of the bone slices with organic solvents or of desiccation *in vacuo* have also been carried out. The results of these have set in sharp distinction two mechanisms in calcifying hypertrophic cartilage, the phosphatase mechanism which can produce in the matrix a local supersaturation sufficiently great to cause deposition of the bone phosphate, and the second, still obscure, mechanism, which favours, and may determine, deposition of the bone phosphate from its supersaturated solution.

Method of experiment.

The experiments were carried out by comparing the calcification *in vitro* of slices cut under sterile conditions from the bones of rachitic rats (fed three to four weeks on McCollum diet 3143). The distal heads of the femora and the proximal heads of the tibiae and humeri were cut longitudinally by hand, 12–14 slices, about 1 mm. thick, being in this way obtained from each rat.

Solutions containing all the salts except the calcium chloride were sterilised in 100 cc. graduated flasks by heating for 30 minutes in steam. The p_H was adjusted by allowing carbon dioxide to diffuse through the cotton wool plug, or by adding sterile acid or alkali when the solution contained no bicarbonate. The requisite amount of sterile calcium chloride solution was then added, the volume made up to the mark and the plugs replaced by rubber stoppers.

In most of the experiments one or more bone slices were placed in each flask and kept at 37° for the stated period. The inorganic phosphate in the solutions was determined at the beginning and end of each experiment. In absence of phosphoric ester the initial and final values did not differ appreciably except in rare cases when general precipitation of calcium phosphate occurred. In presence of phosphoric ester a rise in the concentration of inorganic phosphate was observed. The increase varied with the duration of the experiment, the size of the bone slices and the concentration of ester, and was due partly to hydrolysis of ester in the external solution by phosphatase diffusing out of the bone, and partly to hydrolysis of ester within the bone and subsequent diffusion into the surrounding solution of the inorganic phosphate which escaped precipitation. This increase was usually between 0·1 mg. and 0·5 mg. P per 100 cc., but sometimes exceeded the latter amount when the concentration of phosphoric ester was high. The lower initial level of inorganic phosphate in these "organic" solutions ensured that in spite of this increase the final concentration never rose above that in the corresponding "inorganic" solutions in the same experiment.

A modification of the large constant-flow apparatus previously described was used for experiments in which it was important to avoid any change in the level of inorganic phosphate. With this modified apparatus the calcifying power of twelve different solutions could be compared simultaneously, 100 cc. of each solution being made to flow over a bone slice during a period of about 16 hours at 37°. Each slice was placed in a small bulb tube connected on one side with the vessel containing the solution and on the other, by a length of rubber tubing, with a glass capillary outlet. The twelve outlets, fixed together, were lowered at a constant rate by clockwork mechanism, a continuous flow of solution over each slice being thus maintained. The p_H was kept between 7·3 and 7·4 by equilibrating the solutions with air containing 5 % carbon dioxide, supplied to each vessel from a large aspirator.

When the bone slices were removed from the calcifying solutions they were placed in 10 % neutral formalin (2 hours), washed and placed in 2 % silver nitrate solution (2 hours). After thorough washing in distilled water they were blackened by exposure to light, dehydrated with acetone, cleared in cedar wood oil and examined with low magnification under a binocular microscope. The approximate extent and density of the new deposit is indicated in the tables by a number and by bars, the numbers (maximum 10) showing the area of the zone of hypertrophic cartilage calcified *in vitro*, and the bars (maximum 5) the density of the new deposit. Thus the maximum mark 10≣ assigned to

any slice would indicate that the new deposit extended throughout the entire area and thickness of the zone of hypertrophic cartilage previously uncalcified. One slice from each bone was fixed at the time of cutting and examined to ascertain the extent of the zone and its freedom from patches of calcification due to healing rickets which, if present, invalidated the experiment. In humeri and femora, after immersion in the calcifying solutions, a cap-like zone of newly calcified matrix was sometimes observed in the deeper layers of the cartilage forming the articular surface of the epiphysis, the appearances in this zone reproducing on a smaller scale those seen in the metaphysis. In control slices of these bones the zone of hypertrophic cartilage present in this region was entirely uncalcified. The new deposit in the epiphysis, as seen in thin section, is illustrated in Plate XI, fig. 1. Calcification in this area was not taken into account in marking the extent of the deposit in the experimental slices.

As the size of the different bones and the area of the uncalcified zone varied considerably, endeavour was made to use slices from the same bone for the most critical comparisons in any experiment. Where more than one rat was used the results obtained with bone slices from the different animals are given in separate columns.

Effect of bicarbonate.

The results of six experiments are set out in Table I, showing the effect of bicarbonate in different concentrations on calcification *in vitro*. Four basal solutions, *A*, *B*, *C* and *D* were used, their composition being given at the head of the Table. They were made up in concentrated form and diluted after the addition of phosphoric ester, inorganic phosphate and calcium in the amounts stated, these constituents being added as solutions of sodium glycerophosphate, potassium dihydrogen phosphate neutralised with sodium hydroxide, and calcium chloride prepared from Iceland spar. Solution *A* contained bicarbonate in concentration similar to that in plasma, solution *B* contained the much smaller amount used in previous experiments, while *C* and *D* contained none. As this omission involved a reduction in the sodium content, extra sodium chloride was added to solution *D* to bring the sodium concentration up to that in solution *A*.

The results of these experiments, and of seventeen others not quoted in the table, show that in absence of phosphoric ester calcification regularly takes place at lower concentrations of calcium and inorganic phosphate (that is, at lower levels of the product [Ca] × [P]), when bicarbonate is present than when it is absent. In solutions containing no bicarbonate calcification occurred at 8 : 6 : 0[1] (Exp. 183) but only in one slice out of twelve at 8 : 5 : 0 and never at levels lower than this. In bones from the same animal calcification in presence of 0·03 % bicarbonate was more extensive at the above levels and also occurred at 8 : 4·5 : 0 (Exp. 147). In presence of 0·2 % bicarbonate the extent and density of the new deposit were still greater, while calcification occurred at

[1] The concentrations of calcium, inorganic P and organic P respectively in mg. per 100 cc. are thus indicated throughout the text.

levels as low as 6 : 5 : 0 and 8 : 4 : 0. Calcification failed at 6 : 4 : 0 but was observed at 10 : 2·5 : 0 in one experiment not shown in the table.

Table I.

					Solution *A*	Solution *B*	Solution *C*	Solution *D*
$NaHCO_3$ g. per 100 cc.					0·2	0·03	.	.
NaCl ,,					0·5	0·5	0·5	0·65
KCl ,,					0·03	0·03	0·03	0·03
$MgSO_4, 7H_2O$,,					0·02	0·02	0·02	0·02
		mg. per 100 cc.						
Exp.	Time hrs.	Ca	P inorg.	P org.				
99*	17	6	4	0	0	.	.	0
		6	5	0	1=	.	.	0
		6	6	0	2≡	.	.	0
		6	4	3	2≡	.	.	0
		6	5	3	4≡	.	.	0
114	17	10	4	0	3=	.	.	0
		10	2·5	3	3≡	.	.	0
		10	2·5	10	8≣	.	.	6=
134	17	8	5	0	3=	.	0	0
		8	4·5	3	7≡	.	4≡	4=
					Rat *a* / Rat *b*	Rat *a* / Rat *b*	Rat *a* / Rat *b*	
146	10	8	4·5	0	1= / 3=	. / .	0 / 0	.
		8	5	0	2= / 7≡	. / .	0 / 0	
		8	4·5	1	1= / 7=	. / .	0 / 0	.
		8	4·5	3	5≡ / 9≡	. / .	1= / 4=	.
		8	4·5	10	8≣ / 7≣	. / .	7≡ / 7≣	.
		8	4·5	30	8≣ / 9≣	. / .	8≣ / 9≣	.
147	23	8	4	0	6= / 4=	0 / 0	0 / 0	.
		8	4·5	0	4≡ / 8≡	2= / 3=	0 / 0	.
		8	5	0	6= / 5≡	4≡ / 4≡	2= / 0	.
183	16	8	5	0	5= / 6=	3— / 0	0 / 0	.
		8	6	0	7≡ / 6≡	5= / 5=	2— / 2—	.

* An asterisk after the number of an experiment signifies that the constant-flow apparatus was used.

These results suggest that a less soluble calcium salt is formed in presence of bicarbonate than in bicarbonate-free solutions, and thus provide some evidence that the deposit normally formed in calcifying cartilage is a complex carbonato-phosphate (carbonato-apatite), rather than a mixture of hydroxy-apatite and calcium carbonate.

In presence of phosphoric ester in low concentration calcification occurred at lower levels of inorganic phosphate in all solutions, but the favourable effect of bicarbonate was still noticeable. The effect was masked when the concentration was raised to 30 mg. P per 100 cc., the calcification being then nearly maximal in both solutions (Exp. 146).

Solution *A* was used as the basal solution for all further experiments described in this paper, the necessary modifications being made when the effect of magnesium, potassium or sodium salts was being investigated.

The concentrations of the basic and acidic radicals calculated for the

solution 10 : 4 : 0 are given below. Those of sodium, magnesium and chloride are somewhat below the average values for normal human plasma.

	g. per litre	Millimols per litre
Na	2·53	105·8
K	0·20	5·3
Mg	0·02	0·8
Ca	0·10	2·5
Cl	3·35	87·0
HCO_3	1·45	24·0
SO_4	0·08	0·8
PO_4	0·126	1·3

Effect of sodium chloride.

Shipley and Holt [1927, 1 and 2], using solutions containing inorganic salts alone, found that sodium chloride in concentrations even lower than occur in blood has an inhibitory effect on calcification. Our own experiments confirm this. The results given in Table II show that calcification occurred at the low level 8 : 2·5 : 0 in absence of sodium chloride but was inhibited by this salt in 0·2 % concentration. At 8 : 3 : 0 calcification was obtained with solutions containing 0·2 % sodium chloride but was inhibited by 0·5 %. At higher levels of inorganic phosphate (8 : 4 : 0 and 8 : 4·5 : 0) calcification occurred in all solutions but to a greater extent in those containing 0·2 % sodium chloride than in those containing 0·5 %. In the solutions containing no sodium chloride general precipitation took place at these levels. In presence of phosphoric ester the inhibitory effect of sodium chloride was still discernible but was less marked owing to the much greater calcification obtained in all solutions. This experiment, indeed, shows very clearly the pronounced effect of phosphoric ester on calcification.

Table II.

Basal solution: solution *A* without sodium chloride.

		mg. per 100 cc.			NaCl g. per 10 cc.		
Exp.	Time hrs.	Ca	P inorg.	P org.	0	0·2	0·5
174	16	8	2·5	0	5=	0	0
		8	3·0	0	2=	2=	0
		8	2·5	3	7≡	7≡	6=
		8	2·5	10	9≡	8≡	8≡

Effect of potassium and magnesium salts.

Shipley and Holt [1927, 1] found that the effect of potassium chloride on calcification was similar to, though somewhat less than, that of sodium chloride, but no inhibition was observed with such low concentrations of potassium as are present in plasma. They found, however, that magnesium in very low concentrations (less than 1 *mM*) inhibited calcification, a conclusion also reached by Kramer, Shelling and Orent [1927]. Our own experiments with potassium (Table III) were confined to concentrations not exceeding 10 *mM* (twice the concentration in plasma) and no definite inhibitory effect was observed, either

Page 1931, Table II,
***for* NaCl g. per 10 cc. *read* NaCl g. per 100 cc.**

in inorganic solutions or in presence of phosphoric ester. On the other hand, 2 mg. magnesium per 100 cc. (0·84 *mM*) had an adverse effect on calcification and with twice this concentration inhibition was pronounced in experiments of 5 hours' and 19 hours' duration. It was apparent also in presence of small amounts of phosphoric ester. With higher concentrations of the ester the effect of magnesium was masked.

Attempts to obtain deposits of magnesium phosphate in bone slices by immersion in solutions free from calcium but containing magnesium in high concentration (up to 48 mg. per 100 cc.) were unsuccessful.

Table III.

Basal solution: solution *A* without potassium and magnesium salts.

		mg. per 100 cc.									
Exp.	Time hrs.	Ca	P inorg.	P org.	K 5 / Mg 0	5 / 4	20 / 0	20 / 2	20 / 4	0 / .	mg. per 100 cc.
76	5	10	4	0	3≡	0	3=	2=	1—	.	
		10	3·5	10	7≡	2≡	8≡	7≡	5≡	.	
					K 0 / Mg 0	0 / 2·4	0 / 4·8	19·5 / 0	19·5 / 2·4	19·5 / 4·8	mg. per 100 cc.
150	19	8	4	0	5≡	2≡	0	6≡	4≡	0	
		8	5	0	6≡	6≡	2=	6≡	4≡	0	
151	19	8	3·5	3	3≡	1=	1=	8≡	3=	1=	
		8	3·5	30	9≡	7≡	6=	6≡	9≡	7≡	
					K 0 / Mg 0	19·5 / 0	39 / 0	0 / 2	39 / 2	. / .	mg. per 100 cc.
152	19	10	3	0	3≡	7≡	4≡	.	.	.	
		5	6	0	4≡	4≡	4≡	.	.	.	
		5	8	0	4≡	4≡	5≡	.	.	.	
158	18	10	2·5	3	.	.	.	8≡	3≡	.	
		5	5	3	.	.	.	9≡	5=	.	
		2·5	10	3	.	.	.	0	0	.	
		2·5	10	30	.	.	.	4—	5=	.	

Calcification at different levels of calcium, inorganic phosphate and phosphoric ester.

Table IV shows the calcification obtained with concentrations of calcium between 2·5 and 50 mg. per 100 cc. and of inorganic phosphate between 0 and 20 mg. per 100 cc. in absence of phosphoric ester. It will be seen that the lowest levels for calcification in solutions containing phosphate were 2·5 : 14, 5 : 7, 10 : 3, 20 : 1, giving products of 35, 35, 30, 20 respectively. These results would appear to show that calcification occurs at lower levels of the product [Ca] × [P] when the concentration of calcium is high and that of inorganic phosphate is low than when the conditions are reversed. This is not inherently improbable since, for example, if the deposited salt were $Ca_3(PO_4)_2$, supersaturation of the solution would depend on the product $[Ca^{\cdot\cdot}]^3 \times [PO_4^{\equiv}]^2$. It will be seen that when the concentration of calcium was 20 or more mg. per 100 cc., small deposits were sometimes obtained in solutions containing no phosphate. Presumably these deposits consisted of calcium carbonate unless

phosphate was taken from the bone slice itself, either from the cells or from the calcified portions. The latter possibility, which might be of some interest in connection with resorption and re-deposition of the inorganic material during bone growth, is being further investigated.

Table IV.

Basal solution *A*. 16 hrs.

P mg. per 100 cc.	Ca mg. per 100 cc. 2·5		5		10		20			30		40		50
0	.	.	.	.	.	0	0	1—	0	0	1=	1—	0	2=
1	.	.	.	.	.	.	2—	0	1=	1=	3=	3=	.	2=
1·25	.	.	.	.	.	.	2—	.	.	.	.	.	.	.
1·5	.	.	.	.	.	.	.	+	3≡	.	3≡	+	.	.
2	.	.	.	.	.	.	.	5=	4≡	+	.	.	.	.
2·5	.	.	.	.	.	0	.	8≡	+	.	.	.	.	.
3	.	.	.	.	2—	2=	.	.	.	.	.	.	.	.
3·5	.	.	.	.	3=	4≡	.		.	.	.	.	.	.
4	.	.	.	0	3=	4≡	.	.	.	.	.	.	.	.
5	.	.	.	0	.	4≡	.	.	.	.	.	.	.	.
6	.	.	0	0	.	.	.	.	.	.	.	.	.	.
7	.	.	4—	.	.	.	.	.	.	.	.	.	.	.
8	.	0	3=	5=	.	.	.	.	.	.	.	.	.	.
10	.	0	.	7≡	.	.	.	.	.	.	.	.	.	.
12	0	0	.	.	.	.	.	.	.	.	.	.	.	.
14	1—	.	.	.	.	.	.	.	.	.	.	.	.	.
16	4—	2—	.	.	.	.	.	.	.	.	.	.	.	.
20	.	2=	.	.	.	.	.	.	.	.	.	.	.	.
	a	*b*	*a*	*b*	*a*	*c*	*a*	*d*	*c*	*d*	*e*	*d*	*c*	*e*

Five rats, *a*, *b*, *c*, *d* and *e*, were used in these experiments, the results in any columns being obtained with bone slices from the rat specified at the foot of that column.

+ signifies that general precipitation occurred in the solutions.

Table V.

Basal solution *A*.

Exp.	Time	mg. per 100 cc. Ca	Inorg. P	Glycerophosphoric ester mg. per 100 cc. 0	0·5	1	3
88*	16	10	3	2≡	4≡	7≡	5≡
97*	16	10	3	1=	.	.	4≡
		8	4	1=	.	.	5≡
		6	4	0	.	.	1=
		6	3	0	.	.	1≡

Table V shows the effect of phosphoric ester in low concentrations on calcification at different levels of calcium and inorganic phosphate. Both the experiments here quoted were carried out in the constant-flow apparatus so that the concentration of inorganic phosphate in the solutions surrounding the bone slices did not appreciably alter. At the level 10 × 3, calcification occurred in absence of ester but was definitely increased in presence of glycerophosphate equivalent to only 0·5 mg. P per 100 cc. With 3 mg. organic P the extent and density of the deposit were still greater, while definite calcification was obtained at a [Ca] × [P] level as low as 6 × 3.

The potentialities of the phosphatase mechanism as a factor in calcification have been demonstrated in yet another way, namely, by comparing the minimum time required for calcification, when this mechanism is enabled to function by addition of phosphoric ester to the solution, with that required at higher [Ca] × [P] levels but in absence of ester. The results of experiments with bone slices from four rats are shown in Table VI. Five slices from one rat were placed in a flask containing 40 cc. of the 8 : 5 : 0 solution at p_H 7·35, and five other slices from the same animal were placed in a similar quantity of solution 8 : 4 : 30. The solutions were changed hourly and the inorganic phosphate estimated, no significant increase being found. Single slices were removed at intervals, fixed and examined. The 23 hours' test was carried out separately, one slice being placed in 100 cc. of each solution which was not changed, so that at the end of 23 hours the concentration of inorganic phosphate was very nearly the same (5 mg. per 100 cc.) in both solutions. It will be seen that in presence of ester calcification was obtained in the first two hours. The deposit increased rapidly both in extent and density and was almost maximal in all slices after 10 hours. In the inorganic solutions calcification was first observed in a slice removed after 8 hours and was far from maximal even after 23 hours.

Table VI.

Basal solution *A*.

	mg. per 100 cc.							
Ca	8				8			
P inorg.	5				4			
P org.	0				30			
Time hrs.	Rat *a*	Rat *b*	Rat *c*	Rat *d*	Rat *a*	Rat *b*	Rat *c*	Rat *d*
2	0	.	0	.	3—	.	6—	.
3	0	0	0	.	4=	4=	8=	.
4	0	.	0	0	6=	.	8≡	9≡
5	.	0	.	.	.	5—	.	.
6	0	.	0	0	6≡	.	7≡	9≡
7	.	0	.	.	.	6≣	.	.
8	.	0	.	3—	.	8=	.	9≣
10	4=	0	6—	2=	8≣	8≣	9≣	9≣
23	3≡	0	4≡	5≣	9≣	7≣	10≣	9≣

The results in Table VI illustrate one of the difficulties encountered in these comparative experiments, namely, the variations in the calcification obtained under similar conditions in the bones from different rats. Rats *a* and *b* were from one litter and *c* and *d* from another. All four animals were kept for 24 days on McCollum diet 3143, and all weighed about 60 g. when killed. The exceptional failure of bone slices from rat *b* to calcify in the 8 : 5 : 0 solution is the more interesting since other slices from this rat calcified well in presence of phosphoric ester, showing that the phosphatase mechanism was unimpaired. Small variations in the response of bones from different rats were not infrequent at the levels 30–35 of the product [Ca] × [P], while, at the other extreme, a single case of calcification at the exceptionally low level, 10 : 2·5 : 0, has been

noted. The variations were much less apparent when calcification took place in presence of phosphoric ester.

Effect of hydrogen ion concentration.

Shipley, Kramer and Howland [1926], using inorganic solutions at the [Ca] × [P] level 10 × 3·5, found that calcification did not occur at a p_H lower than 7·0, and Kramer, Shelling and Orent [1926] obtained the same negative result with the higher level 10 × 5. This failure to calcify may, however, have been due to the increased solubility of calcium phosphate in the more acid medium; the solutions, though supersaturated at p_H 7·3, may have been unsaturated at a p_H below 7·0. By using higher [Ca] × [P] levels (10 × 6, 10 × 7, 10 × 8) we have obtained calcification at p_H 6·5, in inorganic solutions, while even better deposits were obtained at lower levels (6 × 4, 8 × 4) in presence of phosphoric ester (Table VII). Between p_H 7·2 and 8·0 we find no marked optimum either in presence or absence of ester, but if the p_H is higher than 7·8 general precipitation often occurs at the [Ca] × [P] levels used in our experiments. The results of four experiments out of a total of fourteen are given in Table VII. The figures in brackets refer to calcification in bone slices which had been previously soaked in acetone, so that only the phosphatase mechanism was able to function. (See the next Section.)

Table VII.

Basal solution *A*.

		mg. per 100 cc.			p_H					
Exp.	Time hrs.	Ca	P inorg.	P org.	6·5	7·1	7·2	7·35	7·5	7·8
121	17	6	6	0	0	.	5=	5=	5=	7=
		6	4	10	2=	.	7=	9≡	7≡	9≡
148	20	8	5	0	0	.	3≡	5≡	5≡	6≡
					0	.	4≡	7≡	7≡	6≡
149	5	8	4	30	Rat *a* 5=	6=	.	8=	8=	7=
					(2)=	(5)≡	.	(6)=	(5)=	(4)=
					Rat *b* 5=	6=	.	5≡	9≡	7=
					(3)≡	(6)≡	.	(6)=	(7)≡	(6)=
185	18	10	6	0	1=	.	.	.	.	.
		10	7	0	4=	.	.	.	.	.
		10	8	0	4=	.	.	.	.	.

In view of the high optimum p_H of bone phosphatase as determined in aqueous extracts, it was at first sight surprising that the p_H of the solutions had so little effect on calcification in presence of ester, especially after the second mechanism had been destroyed by treating the bone slices with acetone (Exp. 149). It is possible that the optimum p_H is not the same for the enzyme acting in the cartilage as for the extract, but a more probable explanation of our results is that, under the conditions of these experiments, the enzyme activity, even at low p_H, was more than sufficient for the hydrolysis of the ester diffusing into the cartilage, and that the rate of diffusion of the ester was the limiting factor governing the amount of calcification obtained.

Effect of chloroform and other organic solvents.

Shipley, Kramer and Howland [1926], in their experiments with inorganic solutions, found that calcification did not occur in bone slices which had been previously soaked in chloroform, 95 % alcohol, ether or toluene, and that it was also inhibited by adding a few drops of toluene to the solution. In the earlier experiments carried out in this laboratory [Robison, 1923; Robison and Soames, 1924] chloroform was usually added to the solutions to guard against bacterial growth, but this did not prevent calcification from taking place. In the two experiments quoted in Table VIII calcification in absence of phosphoric ester, at high [Ca] × [P] levels, was inhibited, though not completely, by saturating the solution with chloroform (about 1 %) and to a less extent by half saturation. In presence of phosphoric ester, however, good calcification was always obtained, though when the concentration of ester was low (3 mg. P per 100 cc.) calcification was slightly less in presence of chloroform than in the control solutions.

Table VIII.

Basal solution *A*.

Exp.	Time hrs.	mg. per 100 cc. Ca	P inorg.	P org.	Chloroform cc. per 100 cc. 0	0·5	1 (saturated)
110	17	10	5	0	7≡	2=	.
		10	6	0	9≡	6=	.
		10	4	3	9≡	7≡	.
		10	4	10	10≡	8≡	.
		10	4	30	10≡	10≡	.
178	21	10	4	0	7≡	.	0
		10	5	0	6≡	.	0
		10	6	0	8≡	.	4=
		10	4	3	6≡	.	4≡
		10	4	10	7≡	.	8≡
		10	4	30	8≡	.	10≡

In the saturated solutions the bone slices were suspended by silk threads to avoid contact with any chloroform globules at the bottom of the flask.

We next investigated the effect of soaking the bone slices for 20 hours at room temperature in chloroform, acetone or 90 % alcohol, before immersion in the calcifying solutions, untreated slices of the same bones being placed directly in similar solutions as controls. The results, shown in Table IX, confirm the findings of the American workers, in that calcification was usually inhibited in inorganic solutions even at high [Ca] × [P] levels. Calcification

Table IX.

Basal solution *A*. 18 hrs. at 37°. p_H 7·4.

mg. per 100 cc. Ca	P inorg.	P org.	Control (untreated) Rat *a*	Rat *b*	Rat *c*	Rat *d*	Acetone Rat *a*	Rat *c*	Rat *d*	90 % alcohol Rat *b*	Rat *c*	Rat *d*	Chloroform Rat *c*	Rat *d*
8	5	0	2—	1—	6≡	6≡	0	0	1=	0	2=	0	0	0
8	6	0	4=	2=	7≡	7≡	0	0	0	0	0	3=	3=	0
8	4	10	6≡	3≡	.	.	8≡	.	.	5≡	.	.	.	.
8	4	30	7≡	7≡	9≡	9≡	8≡	9≡	9≡	9≡	9≡	9≡	9≡	9≡

did, however, take place in a few bone slices, though the deposits were much smaller than in the corresponding controls.

In presence of ester very different results were obtained, calcification being apparently unaffected by the previous drastic treatment of the bones. Heavy deposits were obtained in all slices, not only in the four experiments quoted, but also in many others of the same type. These deposits when examined in the stained slices, or in sections of these cut with the freezing microtome, without previous decalcification, appeared remarkably similar in character to the deposits in the control slices. Photomicrographs of five sections are shown in Plate XI.

Essentially the same results were obtained with slices dried *in vacuo* over sulphuric acid at room temperature for 24 hours before immersion in the calcifying solutions. The slices, though much shrivelled when first placed in the solutions, regained their former size in about half an hour. As shown in Table X calcification in inorganic solutions was inhibited in all but one slice, while in presence of ester normal deposits were obtained. Direct phosphatase tests on bone slices similarly desiccated showed that the enzyme activity was unimpaired by this treatment.

Table X.

Basal solution *A*. 17 hrs. at 37°. p_H 7·4.

mg. per 100 cc.			Control (untreated)			After desiccation				
							Rat *b*		Rat *c*	
Ca	P inorg.	P org.	Rat *a*	Rat *b*	Rat *c*	Rat *a*				
8	5	0	2—	6≡	6≡	0	0	0	0	0
8	6	0	4=	5≡	6≡	0	3=	0	0	0
8	4	10	.	5≡	6≡	.	3=	.	5≡	5≡
8	4	30	6≡	9≡	9≡	6≡	7≡	7≡	9≡	7≡

Table XI shows results obtained when formalin or potassium cyanide was added to the calcifying solutions. Formalin in 10 % concentration (4 % formaldehyde) inhibited calcification both in presence and absence of ester. Potassium cyanide in 0·001 *M* or 0·01 *M* concentration prevented calcification in inorganic solutions but not in those containing ester. With low concentrations of the latter the amount of deposit was somewhat reduced in presence of cyanide, but with higher concentrations this slight inhibition was no longer apparent. The different results in presence of these two agents can be understood when their effects on the activity of bone phosphatase are compared. The values at the foot of Table XI show the activities of two phosphatase preparations in presence of formalin and potassium cyanide, as measured by the hydrolysis of glycerophosphate and expressed as percentages of the hydrolysis in the control solution.

Bone slices soaked for 24 hours in neutral 10 % formalin also failed to calcify on subsequent immersion in inorganic solutions, but slight calcification was usually obtained in solutions containing ester. This indicated that the bone phosphatase could not have been completely destroyed, a supposition

confirmed by direct enzyme tests on half bones after similar treatment with formalin, which showed that about 15 % of the phosphatase activity was still retained.

Table XI.

mg. per 100 cc.			Control		Formalin		Potassium cyanide		
							0·001 *M*		0·01 *M*
Ca	P inorg.	P org.	Rat *a*	Rat *b*	1 %	10 % Rat *a*	Rat *a*	Rat *b*	Rat *b*
8	4	0	0	1—	.	0	0	0	0
8	5	0	1≡	1=	.	0	0	0	.
8	4	3	3≡	4≡	.	0	1=	5≡	1≡
8	4	30	7≡	8≡	.	0	6≡	6≡	8≡
Relative activity of bone phosphatase:									
Preparation *A*			100		25	0	100		33
„ *B*			100		28	0·4	93		58

The fact that bone slices, after treatment with organic solvents or desiccation *in vacuo*, will subsequently calcify in solutions containing phosphoric ester, but not in inorganic solutions even of high [Ca] × [P] levels, sharply distinguishes the two mechanisms in calcifying hypertrophic cartilage; (*a*) the phosphatase mechanism which produces in the matrix fluid, by hydrolysis of phosphoric ester, a condition of supersaturation with respect to the bone phosphate, and (*b*) the second mechanism which favours the deposition of the bone salt from supersaturated inorganic solutions, whether the condition of supersaturation in the cartilage matrix is produced by enzymic hydrolysis of phosphoric ester, or by allowing an external supersaturated solution to diffuse into it.

The nature of the second factor is as yet unknown; a slight increase in the p_H of the matrix fluid, brought about by some membrane equilibrium, or the specific affinity of certain groups in the matrix colloids for some constituent of the bone salt might perhaps account for its action. In some respects it resembles a trigger mechanism, releasing from solution the excess bone salt present in the supersaturated fluid. We have provisionally termed it the "inorganic" mechanism without reference to its nature but because it is responsible for calcification in inorganic solutions.

It is injured by potassium cyanide, by fat solvents and by desiccation of the cartilage, all of which leave the phosphatase mechanism unimpaired. It is probable that the differences in response noted in bones from different rats were due to variations in the efficiency of the "inorganic" mechanism.

Our experiments show that either of these two mechanisms could of itself account for the calcification of cartilage *in vivo*, provided that the conditions necessary for it to function were present. These conditions are, for the phosphatase mechanism, that the plasma should be saturated with respect to the bone salt and contain a hydrolysable phosphoric ester, and for the "inorganic" mechanism, that the plasma should be supersaturated with respect to the bone salt. No satisfactory evidence has, however, been adduced that the

plasma is thus supersaturated. The apparent similarity between the concentrations of calcium and inorganic phosphate adequate for calcification in experiments with inorganic solutions and those in normal plasma is misleading, owing to the absence from the experimental solutions of the protein and other organic constituents which greatly depress the ionisation of calcium in plasma. On the other hand the plasma is known to contain small amounts of a phosphoric ester hydrolysable by the bone phosphatase [Martland and Robison, 1926]. It is therefore reasonable to suppose that both mechanisms play a part in normal calcification of the skeleton, the phosphatase mechanism producing the necessary degree of supersaturation in the matrix and the second ("inorganic") mechanism assisting in the deposition of bone salt, in some way not as yet understood.

The experiments here recorded deal exclusively with calcification in cartilage, but the presence of phosphatase has also been demonstrated in membrane bone [Martland and Robison, 1924; Fell and Robison, 1930] and we have recently obtained evidence of calcification *in vitro* in membrane bone, using the parietal bone of rachitic rats. These experiments and others on calcification in solutions containing protein will be described in a subsequent paper.

Summary.

1. The effect of various factors on calcification *in vitro* has been investigated, using the experimental material and technique previously described.

2. In inorganic solutions with salt concentrations approximating to those in plasma, calcification was obtained with concentrations of calcium varying from 2·5 to 50 mg. per 100 cc. and of inorganic phosphate varying from 1 to 20 mg. per 100 cc. Calcification was observed at somewhat lower levels of the product [Ca] × [P] when the concentration of calcium was high and that of phosphate low than under the reverse conditions.

3. With concentrations of calcium around 10 mg. per 100 cc. some degree of calcification was usually obtained in 16 hours when the concentration of inorganic phosphate was such that the product [Ca] × [P] was equal to 30. Variations were, however, observed in the minimum level required with bones from different rats. This variability in response was apparent also in the extent of the deposit obtained at higher [Ca] × [P] levels.

4. Definitely higher [Ca] × [P] levels were necessary for calcification when the concentration of sodium bicarbonate was reduced from 0·2 % to 0·03 %, and still higher levels were required when the bicarbonate was entirely omitted. This suggests that the compound normally deposited in calcifying cartilage is a complex carbonato-phosphate (carbonato-apatite) rather than a mixture of basic phosphate and carbonate.

5. As observed by other workers, sodium chloride in concentrations as low as 34 *mM* (0·2 %) was found to have an inhibitory effect on calcification in inorganic solutions.

6. Potassium chloride in concentrations up to 10 *mM* (twice the concentration in plasma) showed no appreciable inhibitory effect.

7. Magnesium in concentration of 0·08 *mM* (slightly lower than that in plasma) adversely affected calcification, and with higher concentrations the inhibitory effect was pronounced.

8. The inorganic solutions in which calcification occurred were all supersaturated with respect to the calcium salt deposited. In presence of phosphoric ester, however, calcification occurred at [Ca] × [P] levels much lower than 30, while at higher levels the effect of the ester was seen in the greatly increased extent and density of the deposit.

9. The increased calcification which occurred in presence of phosphoric ester tended to mask the favourable effect of bicarbonate and the inhibitory effects of sodium chloride and magnesium. There was no evidence that any of the inorganic radicals directly influenced the enzyme mechanism.

10. Between p_H 7·2 and 7·8 no marked optimum was found for calcification in presence or absence of ester. Calcification was obtained at p_H 6·5 with very high [Ca] × [P] levels (60–80) in inorganic solutions, and at the low level 24, in presence of phosphoric ester.

11. Formaldehyde completely inhibited calcification in inorganic solutions and almost completely inactivated the phosphatase mechanism.

12. The presence of chloroform or potassium cyanide strongly inhibited calcification in inorganic solutions, but had no appreciable effect in solutions containing phosphoric ester.

13. Bone slices, after being soaked for 24 hours in chloroform, acetone or alcohol, or desiccated *in vacuo* over sulphuric acid, failed to calcify on subsequent immersion in inorganic solutions of high [Ca] × [P] levels. In solutions containing phosphoric ester, however, extensive calcification occurred and the deposits were normal in appearance.

14. The results of these experiments set in sharp distinction the two mechanisms in calcifying hypertrophic cartilage.

(i) The phosphatase mechanism which produces in the matrix fluid a condition of supersaturation with respect to the bone phosphate, and

(ii) The "inorganic" mechanism which favours deposition of this salt from supersaturated solutions.

The part played by these two mechanisms in the normal calcification of the skeleton is discussed.

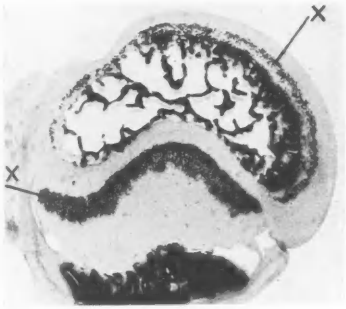

Fig. 1

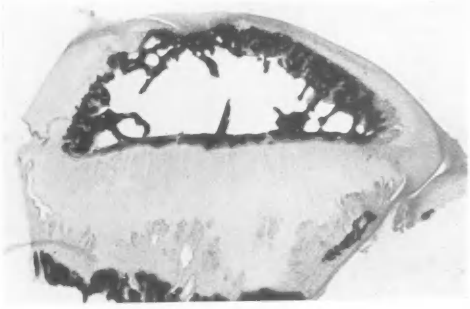

Fig. 2.

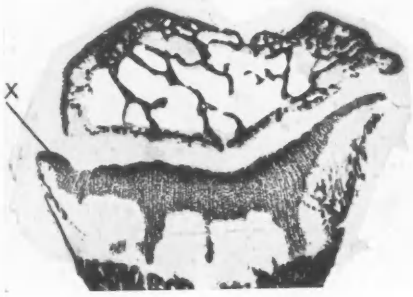

Fig. 3.

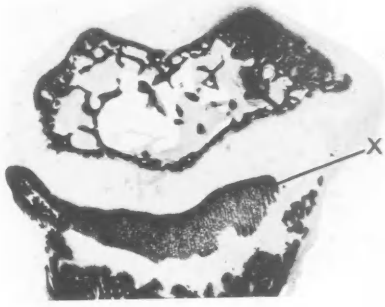

Fig. 4.

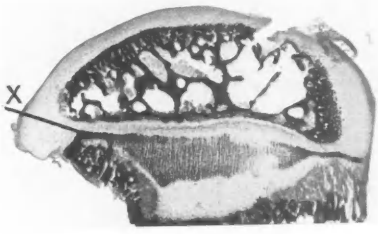

Fig. 5.

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DESCRIPTION OF FIGURES IN PLATE XI.

Figs. 1–5. Sections of bone slices after immersion in experimental solutions at 37° and p_H 7·35. The sections were cut without previous decalcification. Thickness—sections 1, 2, 3, 5—30μ; section 4—50μ. Sections 1, 2, 3 and 4 were stained with silver nitrate, section 5 was stained with purpurin. Magnification × 11. X denotes hypertrophic cartilage calcified *in vitro*.

Fig. 1. Humerus of rachitic rat. 18 hrs. in inorganic solution (8 : 5 : 0). Note the calcification, obtained *in vitro*, in the metaphysis and in the cartilage forming the articular surface of the epiphysis.

Fig. 2. Tibia of the same rat. 22 hrs. in acetone before immersion for 18 hrs. in inorganic solution (8 : 5 : 0). Note the complete absence of new calcification in the broad zone of uncalcified hypertrophic cartilage characteristic of rickets.

Fig. 3. Tibia of the same rat. 18 hrs. in solution containing phosphoric ester (8 : 4 : 30). Note the extensive calcification, obtained *in vitro*, in the matrix of the hypertrophic cartilage.

Fig. 4. Tibia of the same rat. 22 hrs. in acetone before immersion for 18 hrs. in solution containing phosphoric ester (8 : 4 : 30). Note the extensive calcification, obtained *in vitro*, in the matrix of the hypertrophic cartilage, in spite of the drastic treatment of the bone slices. The greater density of the deposit, as compared with that in Fig. 3, is accounted for by the greater thickness of the section.

Fig. 5. Tibia of a rachitic rat. 22 hrs. in 90 % alcohol before immersion for 18 hrs. in solution containing phosphoric ester (8 : 4 : 30). Note the normal appearance of the new deposit in the cartilage matrix.

CCXVIII. THE REACTION OF ANTIMONY TRICHLORIDE WITH COD-LIVER OIL AND ITS UNSAPONIFIABLE FRACTION.

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During the last two years we have had occasion to carry out the antimony trichloride colour test on a number of batches of unsaponifiable matter extracted from fish-liver oils. The oils were saponified by the cold process, or sometimes by boiling with alcoholic sodium or potassium hydroxide, and the unsaponifiable matter was extracted from the soap solution, usually with ether. Although no special precautions (such as working in an atmosphere of nitrogen) were taken to conserve the chromogen[1], it frequently happened that extracts, both those prepared on a small laboratory scale and those prepared on a factory scale, gave values of well over 100 for the percentage extraction, as judged by the colour test. We were aware from published work and from our own observations that the intensity of the blue colour was not a linear function of the concentration of cod-liver oil in the test solution. We had also found that the unsaponifiable fraction gave a linear relationship. This suggested the presence of some saponifiable substance in the oil which inhibited the production of the blue colour—a suggestion confirmed by the observation that the addition of olive oil to the solution of unsaponifiable matter reduced the intensity of the blue colour[2]. Lack of time however prevented until recently a detailed investigation of these phenomena. Meanwhile Norris and Church [1930, 1], working along similar lines to us, reached the same conclusion. They found that oleic acid and unsaturated oils produced the inhibitory effect, and they showed that unsaponifiable matter extracted from cod-liver oil gave an intensity of blue proportional to concentration in chloroform solution, but exhibited the "inhibition" curve typical of cod-liver oil on addition of oleic acid. Norris and Church [1930, 2] conclude that the colour test on cod-liver oil only gives a valid assay if the slope of the tangent to the dilution curve at the origin is measured or calculated. This tangent should coincide with the dilution curve for the unsaponifiable fraction of the corresponding oil (con-

[1] Throughout this paper, the substance present in cod-liver and similar oils, giving a blue colour with antimony trichloride, is referred to as "the chromogen," without prejudice to the question of its identity with, or relation to, vitamin A.

[2] Incidentally, some olive oils, which we have examined, themselves slowly develop a blue colour with antimony trichloride in chloroform.

centrations being expressed in terms of the equivalent amount of oil), but Norris and Church did not secure complete extraction without loss of chromogen, so that their dilution curves for the unsaponifiable fractions cross the curves for the corresponding oils.

Norris and Church used light petroleum for some of their extractions; this solvent is notoriously inefficient for the extraction of unsaponifiable matter from soap solutions in the case of fish-liver oil soaps [bibliography, Smith, 1928; 1]. Most of the data are given for one of their extractions with this solvent [Norris and Church, 1930, 1]; if it may be assumed that the same volume of solvent was used for the second, third, and fourth extractions as for the first (for which it is given), then we are confident that they can only have extracted less than half the total unsaponifiable matter. Very large volumes of solvent and repeated extraction are necessary to secure even approximately complete extraction with light petroleum. In the experiment quoted, Norris and Church used (presumably) 600 cc. of this solvent to extract the soap derived from 100 g. of cod-liver oil; in one of our experiments the same volume of solvent (in 7 portions) was used to extract the soap from only 5 g. of cod-liver oil, and, even so, calculations indicated only 94 % extraction.

The other solvent used by Norris and Church was ethylene dichloride, following the method of Marcus [1928]. Our own experiments have shown that the partition coefficient for unsaponifiable matter between ethylene dichloride and potassium soap magma saturated with ethylene dichloride is very low; calculations showed that to extract about 96 % of the unsaponifiable matter, the soap from 25 cc. of cod-liver oil would need to be treated with about 2500 cc. of ethylene dichloride in four portions. We learn that Norris and Church [private communication] used even larger proportions of solvent than this, but they add, "We were thoroughly aware that the extraction was not complete."

It is true that most of our measurements of partition coefficients were carried out on the total unsaponifiable fraction and not on the chromogen, for which the value is not necessarily the same. So far as our experiments have gone however they have revealed no significant differences between the partition coefficients for the solid (sterol) and liquid fractions of the unsaponifiable matter and for the chromogen, although we have found different values for substances of quite different compositions which have been examined, such as aniline and an azo-dye. It is hoped to extend and publish this work at a later date.

It thus seems evident that Norris and Church did not extract the whole of the chromogen from their saponified oil. This is apart from any destruction which may have occurred—and in this connection it may be mentioned that their use of calcium chloride for drying some of the extracts is open to criticism, since Jones [1928] has shown that calcium chloride may accelerate the destruction of the chromogen by light and oxygen. Norris and Church were unfortunate in their choice of solvents, since with some other solvents, particularly

with ether and ethyl acetate, there is no difficulty in securing practically complete extraction of unsaponifiable matter. We were interested therefore to see whether it would be possible to extract the chromogen without loss by oxidation or thermal destruction during the manipulations involved.

In our first experiment, an elaborate apparatus was set up to enable the whole series of operations to be carried out in an atmosphere of nitrogen. Saponification was effected by boiling for 5 minutes only with strong alcoholic potassium hydroxide, since one of us [Smith, 1928, 1] had previously shown that this was enough for complete saponification; four extractions were made with ether, the ethereal solution was washed, and the bulk of the ether was distilled off in an atmosphere of nitrogen; chloroform was then added, and the distillation was continued at reduced pressure, to remove the remainder of the ether and to dry the extract, traces of water distilling over with the chloroform. When this extract was submitted to the antimony trichloride test, the dilution effect was represented by a straight line, tangential at the origin to the dilution curve for the corresponding oil, as nearly as could be estimated graphically (see Fig. 1).

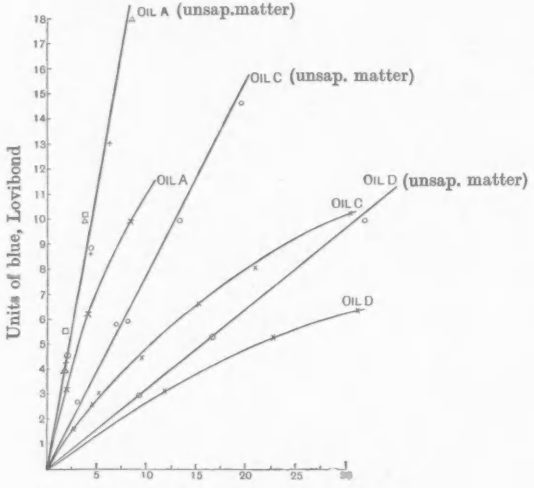

Fig. 1.

× ——— × Cod-liver oil.
○ ——— ○ Unsaponifiable matter extracted by chloroform.
\+ ——— + ,, ,, ,, ether (in nitrogen).
△ ——— △ ,, ,, ,, ether (5 mins. saponification).
□ ——— □ ,, ,, ,, ether (1 hour saponification).

The experiment was repeated without special precautions to exclude air and gave results identical within the limits of experimental error. Another repetition under the same conditions, except that the time of saponification

was increased to 1 hour, again gave complete recovery of chromogen—despite the fact that the ether extract was distilled to dryness and allowed to remain on the water-bath for about 5 minutes before the chloroform was added.

The unsaponifiable matter from 2 g. of cod-liver oil was allowed to form a thin film covering the bottom of a flat-bottomed flask, by evaporation of its solution in chloroform; after solvent vapour had been blown off, the film was heated in an electric oven at 102° for 40 minutes. Even this drastic treatment destroyed only about half of the chromogen originally present.

It appears that the chromogen is more stable than has sometimes been supposed, and that there is no particular difficulty in obtaining practically complete extraction of the total unsaponifiable matter from cod-liver oil. It is nevertheless preferable to avoid exposure of the hot extract to the air, and we always fit the distilling flask with a tap-funnel, so that chloroform may be added without allowing access of air.

Extraction of four different samples of saponified cod-liver oil, with ether, ethyl acetate and chloroform, gave similar results in each case; these are presented in graphic form in Fig. 1. In no case is there any tendency for the dilution curve of the extract to cut that of the oil, indicating that extraction is quantitative within the limits of error of the method. Oil *A* was exceptionally rich in chromogen, oils *B* and *C* were good, while oil *D* was rather poor in this respect, so that these oils are representative of any that are likely to be encountered.

In order to find out whether complete extraction could be secured with light petroleum the following experiment was carried out. 5 g. of cod-liver oil *E* were saponified with 2·5 cc. of 10*N* aqueous potassium hydroxide and 20 cc. of alcohol; 10 cc. of water were added, and alcohol to 50 cc.; the soap solution was purposely made strongly alcoholic, since experiment has shown that this tends to increase the partition coefficient for unsaponifiable matter between light petroleum and soap solution; on the addition of light petroleum, the volume increased to 60 cc. The mixture was extracted with two 50 cc. portions of light petroleum (B.P. 40–60°), the extracts were washed with water, the solvent was distilled off, and a chloroform solution of the residue was tested in the usual way with antimony trichloride. The soap solution was then further extracted with five 100 cc. portions of light petroleum, and the extract was treated in the same way. A greater amount was extracted by this further treatment than by the first, and the sum of the two sets of values yielded a line tangential at the origin to the dilution curve for the original oil (see Fig. 3). Only about 40 % of the chromogen was recovered in the first two extractions, indicating a partition coefficient of about 0·35. From this figure it can be calculated that the whole seven extractions should have removed approximately 94 % of the chromogen originally present in the soap solution. This experiment was repeated using oil *B*, the only difference being that 10 cc. more of water and a correspondingly smaller amount of alcohol were used in preparing the soap solution for extraction. The results, which are included in

Fig. 2, were almost identical with those previously obtained in the proportions extracted by the two treatments with solvent. In addition, the sum of the two sets of values agreed closely with those obtained by extraction with chloroform and with ethyl acetate. Equal volumes of solvent were used for successive extractions, since one of us [Smith, 1928, 2] has shown that with a given volume of solvent and a given number of extractions, the highest degree of extraction is obtained by subdivision of the extracting solvent into equal portions.

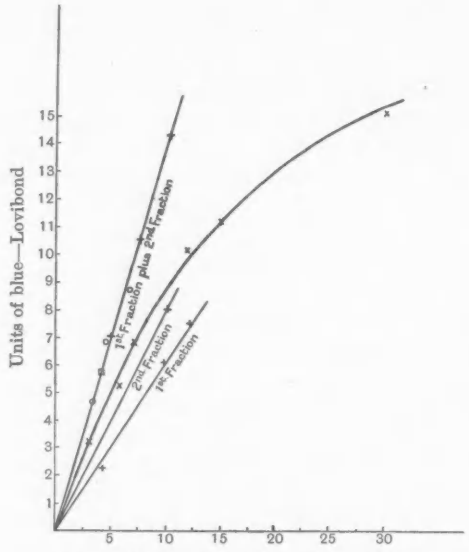

Fig. 2.

× Cod-liver oil.
○ Unsaponifiable matter extracted by chloroform.
\+ " " " light petroleum.
□ " " " ethyl acetate.

A new technique for the antimony trichloride colour test.

Recent workers, particularly Norris and Danielson [1929] and Norris and Church [1930, 1 and 2], have shown that the dilution curves for cod-liver oils are non-linear and vary in shape, so that a true comparison of two oils is not possible when the colour test is carried out in the usual manner. Norris and his colleagues have suggested taking readings at low colour values (below 4 Lovibond units) in which region the dilution curves are approximately linear, or, better, plotting the dilution curves, and comparing the slopes of the tangents at the origin. The tangents are drawn by inspection, or can be calculated in cases where the dilution curve fits a formula of the type $y = ax^2 + bx + c$.

Both these proposed modifications are open to the objection that readings must be taken at very low colour intensities for which the degree of accuracy attainable is, at any rate for the unpractised, considerably less than for readings taken in the neighbourhood of 10 Lovibond units; the second method moreover requires considerable time.

Since we have shown that the chromogen can be readily and quantitatively extracted in the unsaponifiable fraction, which gives a linear dilution curve, an obvious solution of the problem is to carry out the colour test on this fraction.

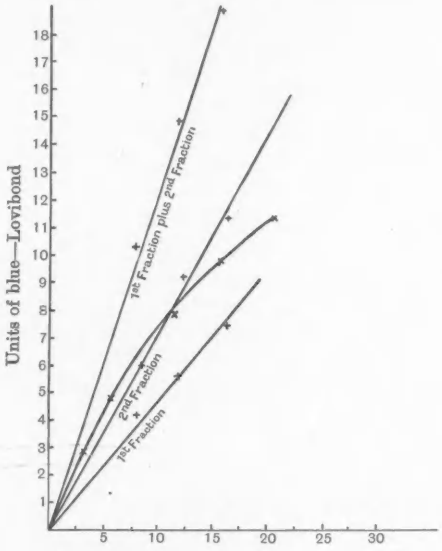

Fig. 3. Cod-liver oil *E*.

\+ Unsaponifiable matter extracted by light petroleum.
× Cod-liver oil.

A just comparison of two samples is then possible from a single determination on each, made near the colour values at which the eye is most sensitive to small variations in blue.

Since the extract is finally required in chloroform solution, chloroform suggests itself for extraction of the soap solution; it is a fairly good solvent for the purpose and does not form emulsions with soap solutions if these contain sufficient alcohol. More reliable results can thus be obtained than by any other method, and certainly in no more time than is required to determine the dilution curve for an oil. The method is described in the experimental section.

Bertram [1929, and private communication], has suggested that it may not be justifiable to evaluate the chromogen content of cod-liver oil by carrying

out the colour test on its unsaponifiable fraction, since any "oxycholesterol" which may be present develops a blue colour with the reagent, though not in the presence of oil. To investigate this point, we prepared "oxycholesterol" by the method of Lifschütz [1919]. The colour given in the test by 0·2 cc. of a 0·4 % chloroform solution of this product (0·8 mg.) was less than 2 Lovibond units, while Rosenheim [1927] obtained for "oxycholesterol" prepared by the action of benzoyl peroxide on cholesterol, 12 units for 1 mg. of the product. Since the unsaponifiable matter from cod-liver oil is known to be composed largely of other substances [Drummond, Channon and Coward, 1925; Drummond and Baker, 1929], it is evident that any trace of "oxycholesterol" which may be present will have a negligible effect on the blue colour developed with antimony trichloride.

The colour developed with cod-liver oil unsaponifiable matter is somewhat difficult to match against Lovibond glasses. Instead of the purplish blue given by cod-liver oil, the colour is almost invariably, in our experience, greenish blue[1] and is matched by a combination of blue and yellow glasses in the ratio of 10 to 4, very approximately. In this observation we are in agreement with Norris and Church [1930, 1]. Moreover, the colour test solution is brighter than the combination of glasses, so that neutral tint glasses have to be used in conjunction with the test solution. In view of the constancy of the colour, we considered the possibility of matching against a permanent colour standard in a colorimeter, as it seemed that this might well give greater accuracy than the Rosenheim-Schuster tintometer. The colour can be imitated by bromocresol green at about p_H 5·4, or by a solution of cobalt chloride in a 0·2 *N* solution of hydrochloric acid in 95 % alcohol. The latter is probably preferable to the solution of a somewhat unstable dye-stuff. Preliminary experiments indicate that colour test solutions could be matched against the cobalt chloride solution in a Hellige type colorimeter with an accuracy at least as great as that attainable with the Rosenheim-Schuster colorimeter.

Experimental.

Ether extraction in nitrogen.

A 350 cc. conical flask was fitted with a reflux condenser and a right-angled tube with stopcock, dipping well into the flask. 10 g. of cod-liver oil were weighed into the flask, which was then flushed out with a rapid current of nitrogen *via* this tube. 5 cc. of 10 *N* aqueous potassium hydroxide and 20 cc. of alcohol were added *via* the condenser, and the mixture was heated on a water-bath for 5 minutes and shaken continuously until the oil globules disappeared. The flask was cooled and 70 cc. of water and 150 cc. of ether were poured down the condenser; after the solution had been shaken and allowed

[1] The only exception was the extract from a sample of oil stored for several years in a half-filled bottle; this, like the oil itself, gave a muddy purple colour with the reagent; the chromogen had been largely destroyed.

to separate, the right-angled tube was adjusted so that its lower end came just above the soap layer, and the ether layer was transferred through this tube to a separating funnel by blowing nitrogen into the flask through the condenser. The separating funnel was equipped with a cork carrying a small tap-funnel for wash liquors and a right-angled tube with stopcock for delivery of the ether extract; it had previously been filled with nitrogen. The extraction was continued with three further portions each of 125 cc. of ether, and the three ethereal solutions were transferred to the same separating funnel. In order to remove soap the combined extracts were washed successively with 10 cc. portions of water, 2*N* sodium hydroxide, water, and dilute hydrochloric acid, and then finally with 200 cc. of water to remove alcohol [Smith, 1928, 1], and transferred to a bolt-head flask previously flushed with nitrogen; most of the ether was distilled off, and chloroform was then added, distillation being continued under diminished pressure until all traces of water had disappeared and the extract was reduced to small bulk. It was then cooled in the atmosphere of nitrogen, and finally rinsed out with chloroform into a 50 cc. graduated flask.

Ethyl acetate extraction.

10 g. of cod-liver oil were mixed with 5 cc. of 10*N* potassium hydroxide and 5 cc. of alcohol, and the mixture was heated on a water-bath for about 5 minutes; 100 cc. of water were added, and the soap was dissolved by gentle heat. Dilute hydrochloric acid was added drop by drop, with stirring, until the soap solution was only faintly alkaline to phenolphthalein. This procedure was adopted in order to avoid hydrolysis of the ethyl acetate by excess of alkali. Four extractions were given, each with 125 cc. of ethyl acetate. The solvent was distilled from the combined extracts under reduced pressure; the residue was then dissolved in about 100 cc. of ether, fatty acid was removed by washing with alkali and water, and the final chloroform solution was prepared as described above.

Chloroform extraction.

The modified colour test. 2 g. of the oil are weighed into a boiling tube; 1 cc. of 10*N* aqueous potassium hydroxide and 5 cc. of 95 % alcohol are added and the mixture is heated in a boiling water-bath for 5 minutes, being shaken until oil globules disappear. The tube is immediately cooled under a tap, and the soap solution is rinsed into a small separating funnel with 25 cc. of water and 10 cc. of alcohol and shaken vigorously with 40 cc. of chloroform. When separation is complete (in about 5 minutes), the lower layer is run into a second separator, and the soap solution is extracted with two further 30 cc. portions of chloroform. The combined chloroform extracts are washed, first with 5 cc. of water to remove most of the soap (shaking must be very gentle, to avoid emulsification), then with two 100 cc. portions of water (the first slightly acidified) to remove alcohol. The chloroform layer is transferred to a small flask, care being taken to prevent globules of water running into the flask,

and the solution is distilled to small bulk; the residue is rinsed with chloroform into a 10 cc. or 25 cc. graduated flask, according to the potency of the oil. The antimony trichloride colour test is carried out on this solution.

In the earlier experiments 35 cc. of chloroform were used for the first extraction and 30 cc. for the second and third. In one case the soap solution was then extracted with three further 25 cc. portions of chloroform. This treatment suffices to recover any chromogen which remains in the soap solution after the first three extractions; the amount recovered was 1/40 of the total. The volumes of chloroform used for extraction were then increased to the values given above, which give a degree of extraction as near 100 % as the method justifies.

Our procedure with the actual colour test differs slightly from that described by others. A suitable weight of the substance to be tested is diluted with chloroform in a small graduated flask. 2 cc. of a solution of antimony trichloride in dry, alcohol-free chloroform saturated at 20° is measured from a burette (fitted with a calcium chloride tube) into a $\frac{1}{2}''$ all-glass cell, and 0·2 cc. of the above solution is run in from a 1 cc. graduated pipette. If added carefully, this floats on top of the denser antimony trichloride solution and the two do not mix appreciably until stirred with a glass rod. Thirty seconds after stirring, the colour is matched by means of a Rosenheim-Schuster tintometer fitted with a daylight lamp. Readings are taken at approximately 20°. Since with unsaponifiable matter the colour intensity-concentration relationship is linear, the value obtained for the chromogen content is not affected by the colour range in which the reading falls; after a preliminary test the concentration of the test solution may be altered if necessary to bring the reading within the range which the observer prefers for colour matching. To compute the units of chromogen per g., we calculate the Lovibond blue units which should be given by 0·2 cc. of a 10 % solution (*i.e.* 20 mg.) of the original substance under the above conditions; for these units we use the symbol β. Thus if the unsaponifiable matter extracted from 2 g. of cod-liver oil is made up to 25 cc. and this solution gives a reading of 6·0 Lovibond units, the chromogen content of the oil is said to be

$$\frac{6{\cdot}0 \times 10 \times 25}{2 \times 100} = 7{\cdot}5\beta \text{ per g.}$$

Summary.

The unsaponifiable fraction of cod-liver oil gives with antimony trichloride in chloroform a blue colour proportional to its concentration.

The line representing the dilution effect for the total unsaponifiable fraction is tangential at the origin to the dilution curve for the corresponding cod-liver oil.

The unsaponifiable fraction can be extracted almost without loss with ether, ethyl acetate, chloroform or light petroleum, under suitable conditions.

A method is described for carrying out the colour test on the unsaponifiable fraction extracted with chloroform.

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CCXIX. THE DETERMINATION OF VITAMIN A.

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It is recognised by all workers in the vitamin field that rats which have ceased to grow on a diet deficient in vitamin A respond to a given dose of that factor to a varied degree. The variations have, in the main, been attributed to variations in the breed of the rats, in the pathological conditions of the animals when considered ready for a test, and in the diet of the colony from which the experimental animals were taken, variations which occurred even when this diet had been kept as nearly uniform as possible. The present paper is an attempt to demonstrate how they may be overcome for the purpose of making a quantitative comparison of the potency of two vitamin-containing substances.

The first experiment.

It has been the practice of many, when testing substances like cod-liver oil, to add one daily dose of the substance to the diet of two or three rats, a second, larger dose to the diet of two or three more rats and a third and even a fourth larger dose to the diet of further small groups of rats. It is generally admitted that the behaviour of each animal does not change strictly according to the size of the dose administered, and that some rats receiving the higher doses resume growth less promptly and grow at a slower rate than those receiving the lower; but it is rarely realised how widely different the responses of different animals may be, so different indeed as to throw suspicion on results with a small group of rats in which there is an appearance of a response graded to the dose. Workers are much inclined, we think, to accept results as satisfactory in an experiment on a few animals in which there is some gradation according to the dose, and to discard others as inexplicable where the gradation is not apparent. There is, of course, no justification for this. We give an example in Fig. 1 which illustrates how different the response of different animals may be. Of two rats prepared in the same way on a vitamin A-free diet containing Glaxo "casein," one rat gained 38 g. in 10 weeks on a daily dose of 2·5 mg. cod-liver oil, whereas the other lost 1 g. in the same time on a daily dose of 15 mg. These irregularities may dominate the picture at the close of the test

so much that it is impossible to detect any gradation of the growth response at all.

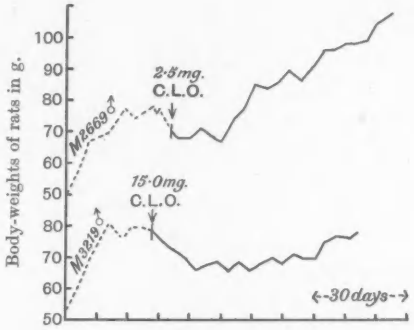

Fig. 1. Exp. 1. Growth curves of two rats showing the wide variation in response to cod-liver oil.

Our first object, therefore, was to determine under what conditions it was possible to obtain a growth response in different groups of animals which should be graded according to the size of the dose of cod-liver oil. It became necessary to find a means of making a sum of the responses of the animals of a group in order to compare it with that of a different group of animals receiving a different dose. The first attempt to do this followed the very simple plan of counting the growth response of each animal as positive or negative according as it had gained 10 g. in 5 weeks or had not. This response is the basis of the definition of the unit of vitamin A activity prescribed by the United States Pharmacopoeia. This plan, though simple, involved a considerable extension of the work ordinarily undertaken in vitamin A examinations. Instead of treating each curve of growth resumption as a result in itself, we proposed to treat it merely as one positive or negative out of a large group of such curves. We, therefore, used approximately 30 animals in each group, each animal in a group being given the same dose of a particular sample of cod-liver oil, which will henceforward be described as oil A. There were in all, in this first experiment, in which Glaxo "casein" was used in the basal diet, 11 groups of about 30 rats each. For every one of the 330 rats, an ordinary weight chart was plotted. The test period from the time when each rat had ceased to grow and when the daily dose of oil A was first administered was 10 weeks.

The diet used in preparing the rats was as follows:

Vitamin-free caseinogen (Glaxo)	15 %
Dextrinised rice starch	73
Dried yeast	8
Salt mixture (Steenbock's 40)	4

This was exposed in thin layers (200 g. over 4 sq. ft.) to the rays of a quartz mercury vapour lamp at a distance of 2 ft. for 30 minutes, the lamp running at 2·5 amps., 130 volts. This generated ample supplies of vitamin D. We made no attempt to divide litters among the different groups. We completed each group as the rats became steady in weight, assuming that they would be as nearly as possible in the same physiological condition when considered by the same workers to be ready for the test. Greater accuracy may be obtainable by putting one animal of each litter into each group, but we have not investigated this point.

In Fig. 2 is shown the extraordinary result which was obtained when

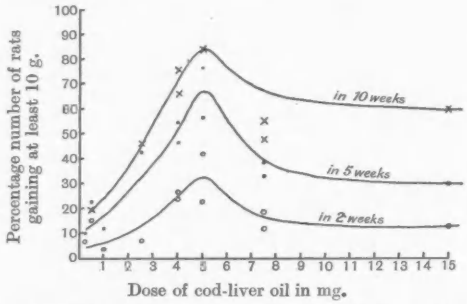

Fig. 2. Exp. 1. The relation of growth response to dose of cod-liver oil. The percentage number of rats in each group that gained at least 10 g. weight in 2, 5 and 10 weeks respectively. "Vitamin-free casein" (Glaxo) in diet.

11 groups, each of about 30 rats, were examined in this way. In Table I is given the order in which the doses were tested. The significance of the points

Table I. *Order and times of testing the different doses.*

Dose in mg.	Date test began	Dose in mg.	Date test began
0·5	Aug. 1928	7·5	July 1929
0·25	Nov. 1928	7·5 (second test)	Aug. 1929
1·0	Feb. 1929	15·0	Sept. 1929
5·0	Apr. 1929	4·0 (second test)	Oct. 1929
2·5	May 1929	5·0 (second test)	Jan. 1930
4·0	July 1929		

on the curves is best explained by an illustration. To each of one group of 28 rats, a daily dose of 2·5 mg. of oil A was administered. Of these rats it was found that two, or 7 %, had gained at least 10 g. at the end of 2 weeks. Twelve rats, or 43 %, had gained at least 10 g. at the end of 5 weeks, and 13 rats, or 46 %, had gained at least 10 g. at the end of 10 weeks. It may be noted at once how little difference there was between the number of rats gaining 10 g. at the end of 5 weeks and at the end of 10 weeks.

We expected, of course, that as the dose of cod-liver oil increased, so the percentage of rats gaining at least 10 g. would increase until the figure of 100 % was reached. Actually the percentage rose, as shown, up to a dose of

5 mg. only. Above this, however, the percentage fell and the dose of 7·5 mg. was apparently less effective than those of 4 and 5 mg. This was an extraordinary result to have reached as a result of an exhaustive research on over 300 animals. At first sight it meant that vitamin A, as present in cod-liver oil, was not an agent which promoted growth in proportion to the amount of it present in the dose. Every estimation of vitamin A which has ever been made, however, assumes that the growth response is, up to a certain limit, dependent on the amount of vitamin. It seemed that some might attempt to explain the observations on the ground that this sample of cod-liver oil possessed some obscure toxic property which had developed as time had passed, and which had interfered with the true effect of the higher doses. Accordingly we tested the doses of 7·5 mg., 4·0 mg. and 5·0 mg. again, and also examined the effect of a daily dose of 15 mg. With slight differences, the previous results were confirmed, and the dose of 15·0 mg. produced an effect not appreciably

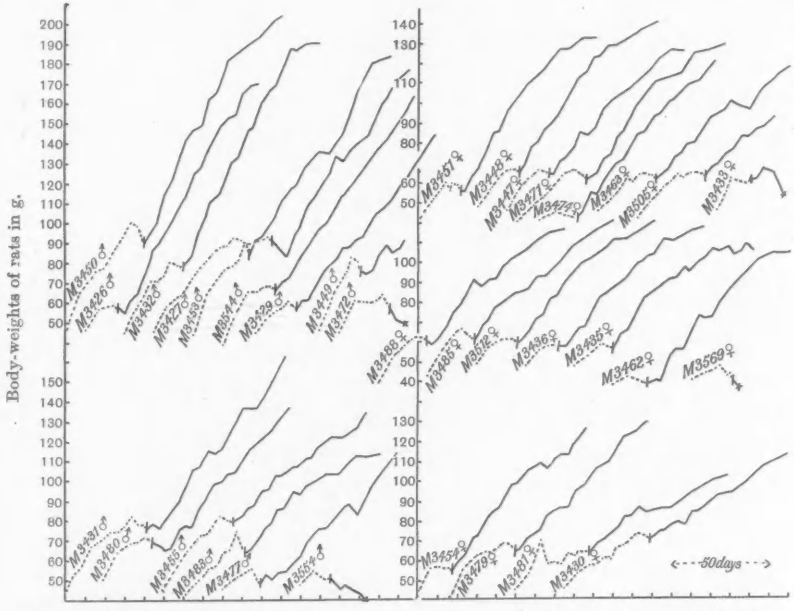

Fig. 3A. Exp. 1. To show the variation in response to a dose of 5·0 mg. cod-liver oil in 34 rats (15 ♂, 19 ♀) during a 10 weeks' test with extracted "light white casein" (B.D.H.) in the diet. The standard deviation in this group was 12·1.

worse than that of 7·5 mg. This excluded, in our opinion, the possibility of any toxic effect. On the other hand it abundantly confirmed the finding that even limited growth was not resumed on this diet by a majority of rats when given an excess of vitamin A.

At this stage of the investigation we had realised from separate experiments

[Coward, Key and Morgan, 1929; Coward, Key, Morgan and Cambden, 1929] that the basal diet described was not always sufficient, together with vitamin A, to produce normal growth, in contrast to a diet in which an extracted caseinogen was used instead of the Glaxo "casein." This extracted caseinogen, which was "light white casein" bought from B.D.H. and extracted by ourselves with alcohol and ether, provided a basal diet on which rats grew rapidly when the diet was supplemented with a small dose of cod-liver oil. Accordingly we prepared a set of rats on a diet similar to the other one in all respects except that it contained "light white casein" (B.D.H.) which we extracted as required once with cold alcohol, and four times with cold ether (200 g. in 1–1·5 litres solvent) in jars with frequent shaking, 24 hours each extraction. As the rats became steady in weight they were used for a test of 5·0 mg. of the oil. The result of this test together with the result on 5·0 mg. on the first diet containing Glaxo "casein" is shown in detail in Figs. 3 A and B. It is

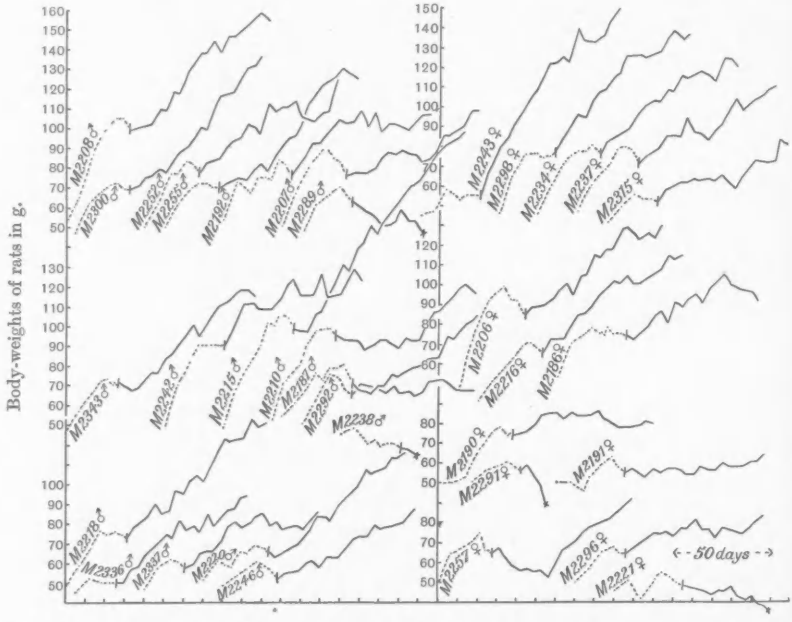

Fig. 3B. Exp. 1. To show the variation in response to a dose of 5·0 mg. of the same oil in 33 rats (19 ♂, 14 ♀) but with "vitamin-free casein" (Glaxo) in the diet. The standard deviation in this group was 16·8.

obvious from a survey of the individual curves that this response is far better than that on the diet containing vitamin-free caseinogen (Glaxo). 93 % of the rats of this group of 34 gained at least 10 g. in 5 weeks, whereas the highest percentage to make such a gain on the other diet was 77 in the first group

that was given 5·0 mg. The results of the first experiment are summarised in Table II. We considered this result additional evidence that the "light white

Table II. *Exp. 1. To show the growth response of groups of rats to different doses of cod-liver oil, "vitamin-free casein" (Glaxo) in the diet.*

Dose of C.L.O. mg.	No. of rats	Date test began	No. dead in 1 week	No. dead in 2–5 weeks	% increasing 10 g. in 5 weeks	Mean increase in 5 weeks in g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n}}$	$\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} . 100$
0·25	29	Nov. 1928	0	4	10	−3	10·3	−0·14100
0·5	29	Aug. 1928	3	3	23	3·5	10·7	0·13866
1·0	36	Feb. 1929	4	3	12	−0·7	11·4	−0·02832
2·5	39	May 1929	11	1	43	4·0	12·4	0·16514
4·0	38	July 1929	8	4	47	10·5	18·3	0·40086
4·0	32	Oct. 1929	3	1	55	12·7	11·8	0·44046
5·0	33	Apr. 1929	2	0	77	21·5	16·8	0·74332
5·0	30	Jan. 1930	1	2	57	12·5	11·3	0·39787
7·5	33	July 1929	0	7	33	10·3	14·1	0·33388
7·5	32	Aug. 1929	1	1	39	7·3	12·0	0·29478
15·0	33	Sept. 1929	3	2	30	6·7	11·0	0·28918
5·0 Cas. B.D.H. in diet	34	Oct. 1929	2	3	93	40·7	12·1	1·3976

casein" has some growth-promoting property which the vitamin-free caseinogen (Glaxò) does not have. As it was clearly impossible to get reliable indications of differences of vitamin A potency by the use of the vitamin-free caseinogen (Glaxo) and the stock of rats that we had available, we decided to repeat the whole experiment, using a diet containing extracted "light white casein" (B.D.H.) instead of the vitamin-free caseinogen (Glaxo), hoping that we should obtain, with some dose, 100 % of the animals of a group gaining at least 10 g. in 5 weeks, and that the curve of response to different doses would be steep enough to give fairly accurate comparisons (say to within 25 or 50 %) between different samples of oil.

The second experiment.

A second sample of the same batch of cod-liver oil was obtained from Messrs Allen and Hanbury and used throughout this experiment with a diet containing extracted "light white casein" (B.D.H.). Doses were tested in the order given in Table III.

Table III. *Order and times of testing the different doses in Exp. 2.*

Dose (mg.)	Date test began	Dose (mg.)	Date test began
2·5	Nov. 1929	0·25	Jan. 1930
7·5	Dec. 1929	1·0	Apr. 1930
20·0	Dec. 1929	1·5	May 1930

The result is plotted in Fig. 4. Curves of the percentage number of rats gaining 20 and 30 g. respectively in 5 weeks are also plotted. The steeper parts of the curves are again plotted in Fig. 5 on a more extended scale. The result is very different from the one obtained in the first experiment

and shows at once that it is possible to get a growth response graded to the dose of vitamin A. Thus, although actual measurements of vitamin A

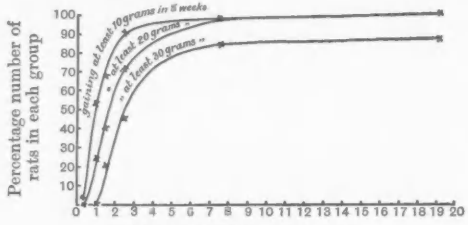
Percentage number of rats in each group
gaining at least 10 grams in 5 weeks
" at least 20 grams "
" at least 30 grams "
Dose of cod-liver oil in mg.

Fig. 4. Exp. 2. The relation of growth response to dose of cod-liver oil. The percentage number of rats in each group that gained at least (*a*) 10, (*b*) 20 and (*c*) 30 g. in 5 weeks. Extracted "light white casein" (B.D.H.) in the diet.

cannot be made without a standard of reference, yet comparisons between different substances with respect to their vitamin A potency may be drawn

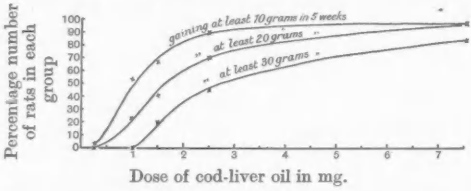
Percentage number of rats in each group
gaining at least 10 grams in 5 weeks
" at least 20 grams "
" at least 30 grams "
Dose of cod-liver oil in mg.

Fig. 5. Exp. 2. Fig. 4 expanded to a more useful form.

with a degree of accuracy equal to that obtained in many assays made by biological methods.

Other methods of measuring the response.

It was obvious that these curves failed to make anything like full use of the data obtained. In Tables II and IV are collected the results of the two experiments, giving the following information.

1. *Number of rats dying during the test.*

The number of rats dying during the first week of the test bears no relation to the dose of cod-liver oil given. Considering the varying and unknown degrees of severity of the pathological conditions of the animals, this is not surprising. These rats have been ignored in all calculations. Some workers discard those that die in the second week also. We have counted those that died in the second to the fifth week, and found in the second experiment a well-marked relation between the number dying during this period and the dose of cod-liver oil given. It is shown in Fig. 6 that it may be possible to devise a method based on a curve of this kind for comparing the vitamin A potency of different substances. The fact that the corresponding figures in Exp. 1 do

Table IV. *Exp. 2. To show the growth response of groups of rats to different doses of cod-liver oil, extracted "light white casein" (B.D.H.) in the diet.*

Dose of C.L.O. mg.	Total no. of rats	Date test began	No. dead in 1 week	No. dead in 2–5 weeks	Rats alive at end of 5 weeks	% increasing 10 g. in 5 weeks	Mean increase in 5 weeks g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n}}$	$\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} .100$
0·25	31	Jan. 1930	2	15	14 (7 ♂, 7 ♀)	3	−11·5	13·75	−0·3583
1·0	37	Apr. 1930	7	6	24 (17 ♂, 7 ♀)	53	13	8·60	0·37837
1·5	35	May 1930	5	3	27 (10 ♂, 17 ♀)	67	17·1	14·8	0·52620
2·5	32	Nov. 1929	1	1	30 (19 ♂, 11 ♀)	90·3	27·7	10·6	0·81349
7·5	31	Dec. 1929	0	1	30 (18 ♂, 12 ♀)	97	48·2 ♂ 55·7 ♀ 37·0	14·3 ♂ 10·3 ♀ 10·4	1·3139 ♂ 1·4846 Init. wt. 81·8 ♀ 1·0391 „ 84·2
20·0	32	Dec. 1929	1	0	31 (15 ♂, 16 ♀)	100	45·4 ♂ 53·0 ♀ 37·2	14·3 ♂ 13·9 ♀ 9·11	1·2349 ♂ 1·3835 Init. wt. 85·1 ♀ 1·0601 „ 83·75

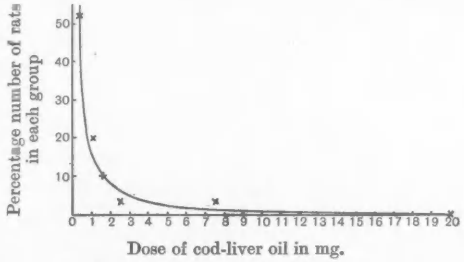

Fig. 6. Exp. 2. The relation between dose of cod-liver oil and the percentage number of rats in each group that died during the second to the fifth week.

not show a similar correlation with dose, would appear to lend additional support to the view that many of the rats in Exp. 1 were not suffering from a deficiency of vitamin A only.

2. *The total growth of all animals of each group* has been expressed in various ways.

(*a*) A composite curve of all the animals of a group may be found by averaging their weights when first given the dose and again at weekly intervals during the test. If a rat dies during the test, the curve may be broken at the week of its death. It ends at the point where it dies, this point including its last weight, and a fresh curve is begun at the same point of time, calculated from the average weight of the remaining rats. The general slope of the curve represents the full growth response of all animals of the group.

The composite curves of all groups of animals in both the experiment with Glaxo "casein" and the experiment with extracted "light white casein" are shown in Fig. 7. They depict quite clearly the differences in response to similar doses obtained by using the different preparations of caseinogen in the basal

diets. The curves obtained by the use of Glaxo "casein" and different doses of oil are very little different on the lower doses, and are practically alike on the higher doses. It would be impossible to make comparisons over the very small range of difference shown by these curves.

It is quite obvious, however, that there are greater differences between the

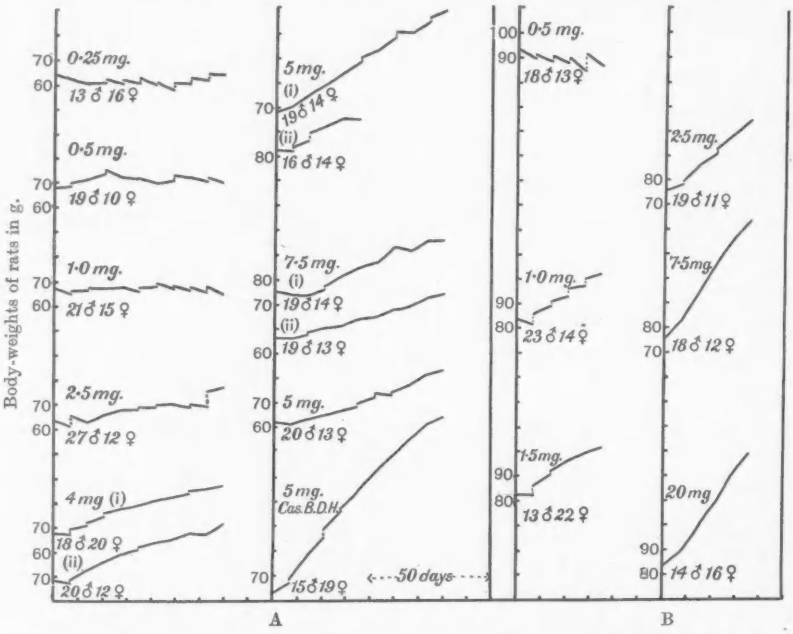

Fig. 7. Composite growth curves of groups of rats on different doses of cod-liver oil. A. Exp. 1. "Vitamin-free casein" (Glaxo) in diet. B. Exp. 2. Extracted "light white casein" (B.D.H.) in diet.

curves obtained by the use of a similar series of doses of oil when extracted "light white casein" is used. The series of curves is useful to indicate the differences in dosage which are responsible for differences in slopes of the composite curves obtained. It is perhaps surprising to note that the dose of oil A (7·5 mg.) which produced the greatest growth (20 mg. produced no more than 7·5 mg.) is, in fact, seven and a half times as great as that (1·0 mg.) which produced slight growth (13 g. in 5 weeks).

(*b*) A curve has been plotted in Fig. 8, relating the mean increase in weight in a group in 5 weeks to the dose administered. It demonstrates very clearly that, up to a point, the growth response increases with the dose of vitamin A given. It appears to be the most direct and complete way of relating response to dose given and, on the lower doses, the slope is steep enough to give results comparable in accuracy with those of most biological measurements. It

ignores differences that may be due to differences in initial weight[1] of animals or groups of animals. Actually, in the experiment, the average initial weights of the groups of animals were 75–85 g.; that is they were sufficiently alike to make it unnecessary to allow for the differences.

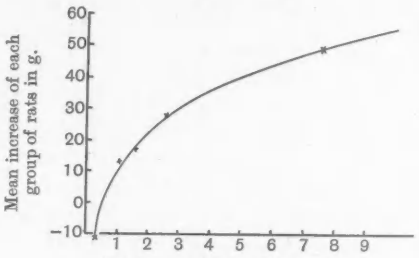

Fig. 8. Exp. 2. The relation between dose of cod-liver oil and the mean increase in weight of each group of rats. Extracted "light white casein" in diet.

The curve drawn in Fig. 8 fits the equation $y = 12{\cdot}1 + 40{\cdot}2 \log x$, which may be used instead of the actual curve for evaluating doses of vitamin A responsible for mean increases in further experiments. The best accuracy would be obtained by giving such a dose as would bring one on to the part of Fig. 8 where the fraction $\frac{\text{standard deviation}}{\text{mean increase} \times \text{slope of curve}}$ is a minimum, which is perhaps at about 20–30 g. mean increase.

(*c*) The curve relating the growth as calculated from the formula

$$\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} . 100$$

to the dose of cod-liver oil is slightly smoother than the curve of mean increase. This formula (in which M_1 is the weight of the group of animals at the beginning of the experiment, M_2 the weight at the end and $(K_2 - K_1)$ the duration of the experiment in days) takes into account the fact that animals grow more slowly as they approach maturity. The curve is shown in Fig. 9.

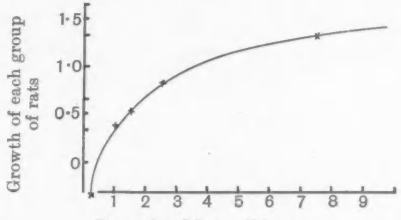

Fig. 9. Exp. 2. The relation between dose of cod-liver oil and the growth of each group as calculated from the formula $\frac{\text{Log}_e M_2 - \text{Log}_e M_1}{K_2 - K_1} . 100.$

[1] The weight at which the rats have ceased to grow on the vitamin A-free diet and have been given the dose of cod-liver oil.

The sequence of testing the different doses is of interest. The fact that the response was graded to the dose, in spite of the doses not having been tested in order of magnitude, indicates that the response was independent of the season at which the test was made. In other words, there is no seasonal variation in the response of animals to the giving of vitamin A.

The degree of accuracy obtainable by the use of smaller groups of animals.

The smoothness of the curve of reference given in Fig. 8 is a general indication of the degree of accuracy attainable by the use of 30 animals on a dose. We may, therefore, accept it as the expression of the true relation between response and dose of vitamin A.

In order to determine the degree of accuracy attainable by the use of a smaller number of animals, we have divided our results on the rats used in the making of the curve into groups of (*a*) 5 animals and (*b*) 10 animals. They were taken strictly in order as they occurred on the record sheets without selection or rearrangement of any kind. The mean increase for each group was calculated and, by reference to the curve, the corresponding dose of cod-liver oil was determined. For example, the mean increases of the different groups of 5 animals each, given 1·0 mg. oil A, were 9, 14, 11·4 and 15·2 g. respectively. By reference to the curve, it was found that the doses of cod-liver oil corresponding to the same mean increases when 30 rats were used in each group were 0·9, 1·2, 1·05 and 1·3 mg. respectively. But the actual dose was always 1·0 mg.; therefore, the percentage errors were 10, 20, 5 and 30 respectively. The figures for all the groups on the various doses are given in Table V.

In the 21 groups of 5 animals each, the errors of one result, and possibly a second, were more than 50 but less than 100 %. The errors of 10 others were 20–50 %, and the errors of the remaining 9 were 0–10 %.

In the 10 groups of 10 animals each, one error was 32 %, three errors were 21–30 %, three were 11–20 % and three were 0–10 %.

Two further results of interest in this connection are quoted in Table VI. Two oils, B and C, were tested by this method, each in two different doses. The figures for oil C are particularly striking. 19 rats given a dose of 1 mg. oil C increased in weight in 5 weeks by an average amount of 22·5 g. But this mean increase would have been given by 1·95 mg. of the oil A (see curve, Fig. 8). Hence oil C is 1·95 times as potent as oil A. A second test of oil C, on a dose five times as great, gave in 17 rats a mean increase of 56 g. in 5 weeks. This increase would have been given by 10·2 mg. of oil A. Hence oil C is $\frac{10{\cdot}2}{5{\cdot}0} = 2{\cdot}04$ times as potent as oil A. Here are two estimations of oil C in terms of oil A, which differ by only $\pm$ 2·5 %, a degree of accuracy as great as that of any other biological estimations.

Thus our actual results showed a degree of accuracy (*a*) generally within 50 % (19 times out of 21) of the true value by the use of 5 rats on a test,

Table V. *To show the variation in result obtained by using smaller groups of animals.*

Each group of 30 animals used in the making of the curve has been divided into groups of (*a*) 5 and (*b*) 10 animals. By referring the mean increase of each small group to the curve, the dose apparently given to produce this increase is found. It is called the "found" dose. Its difference from the actual dose is stated as % error.

	5 animals in each group				10 animals in each group			
Actual dose mg.	Group	Mean increase g.	Found dose mg.	% error	Group	Mean increase g.	Found dose mg.	% error
1·0	1	9·0	0·9	−10	1	11·5	1·05	+ 5
	2	14·0	1·2	+20	2	13·3	1·2	+20
	3	11·4	1·05	+ 5				
	4	15·2	1·3	+30				
	5 (4 only)	16·25	1·4	+40				
1·5	1	19·8	1·65	+10	1	19·1	1·65	+10
	2	18·4	1·55	+ 3·3	2	20·3	1·75	+16·7
	3	29·2	2·8	+87				
	4	11·4	1·05	−30	3 (7 only)	9·6	0·95	−37
	5	12·2	1·1	−27				
	6 (2 only)	3·0	0·6	−40				
2·5	1	27·6	2·5	0	1	29·4	2·9	+16
	2	31·2	3·1	+24	2	32·6	3·3	+32
	3	31·2	3·1	+24	3	21·2	1·85	−26
	4	34·0	3·55	+42				
	5	26·2	2·4	− 4				
	6	16·2	1·4	−44				
7·5	1	42·6	5·6	−25·3	1	42·1	5·5	−26·7
	2	41·6	5·35	−28·7	2	47·9	7·2	− 4·0
	3	49·4	7·75	+ 3·3	3	54·6	9·7	+29·3
	4	46·4	6·8	− 9·3				
	5	49·2	7·7	+ 2·7				
	6	60·0	12·0?	+60·0?				

Table VI. *To show the close agreement of results obtained by testing two different doses of an oil and referring the mean increases of the groups to the curve of the original oil.*

Oil	No. of rats	Actual dose mg.	Mean increase of wt of group in 5 weeks g.	Dose of oil A which produced the same mean increase mg.	Potency of oil in terms of oil A %
B	19	1	7·7	0·78	78
	9	4	29·5	2·83	71
C	19	1	22·5	1·95	195
	17	5	56·9	10·2	204

(*b*) always (10 times out of 10) within 33 % by the use of 10 rats, and (*c*) within 2·5 % (2 out of 2 times) by the use of about 20 rats. The data are too few for us to make a suggestion of the probable error of any determination with any given number of animals. We have calculated the standard deviation of the individuals in each group of 30 animals according to the formula $\sigma = \sqrt{\frac{\Sigma d^2}{n}}$,

where σ is the standard deviation of the individuals, d is the difference from the mean of the group, and n is the number of animals in the group.

These calculations are included in Tables II and IV. In order to determine the standard deviation of the mean of the whole group we applied the formula

$$\epsilon_m = \sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

This is a formula which has been used in many biological estimations, the probability being (*a*) 2/3 that a similar estimation on a similar group of animals would give a result within $\pm \epsilon_m$ of the truth, and (*b*) 21/22 that the result would be given within $\pm 2\epsilon_m$ of the truth. When we applied this formula to our small groups of 10 animals, we found that we should have to expect a result 1 out of 22 times either 50 % below or 100 % above the real dose. Actually we have found a much greater degree of accuracy than this. All the 10 results on the 10 groups of rats fell within 33 % of the true dose, but, after all, with only 10 groups no great stress can be placed on this result.

Irwin, Brandt and Nelson [1930, 1] have lately reported an analysis of results obtained in their laboratory which it is of interest to note here. They have accepted the contention that two mean increases differ significantly only if the difference between them is three times the standard deviation of the difference, and from this they have determined that, in their colony, the difference between two means must be at least 20·45 g. to be significant, and that in order to get this difference each group must consist of 20 animals. They have not, however, examined their figures with a view to discovering whether a smaller difference than three times the standard deviation of the difference between the means is significant, and it seems probable, from their statement concerning the source of their figures, that they have not had a sufficient number of animals on one dose of one particular substance to make such an analysis possible.

Discussion.

Three points which have emerged from the work here described require some discussion. In the first place we have found that it is impossible to make any estimation of vitamin A when Glaxo "vitamin-free casein" is used. It is unsafe, of course, to say that this finding must hold good for other laboratories, for differences in the basal diet or in the diet of the breeding colony may make good the deficiency that we have detected in ours. We suspected the existence of this deficiency after 6 months' use of Glaxo "casein" in these laboratories and found within a few months that it could be made good by the substitution of "light white casein," but it has taken nearly 3 years to show that the inclusion of "light white casein" in our basal diet makes possible a reasonably accurate estimation of vitamin A. It is quite possible to get large numbers of results on single rats like those shown in Fig. 3 B which might lead anyone to conclude that the basal diet used was the best one available, but we believe that the only way to make certain of

this is to demonstrate it by some means such as we have used and depicted in Figs. 5, 6, 8 and 9.

The second point which requires comment is the demonstration that a relation exists between the size of the dose administered to a group and the percentage of animals dying in the period from the second to the fifth week of the administration of cod-liver oil. We believe the results shown in Figs. 6 and 8 indicate that there is the closest relation between the growth-promoting power of vitamin A and the anti-infective power as described by Green and Mellanby [1929], and that a measure of the one is a measure of the other. The essence of the view put forward by Green and Mellanby was that since rats, dying on a shortage of vitamin A, die suffering from infections in different parts of the body, it is to be concluded that the absence of vitamin A means the absence of an anti-infective vitamin on which the resistance of the body depends. We have found that when rats have ceased to grow on a vitamin A-free diet, the addition of small daily doses of cod-liver oil to the diet does not prevent some rats from dying during the next 5 weeks, while it enables others to resume some growth. The rats which die, when examined *post mortem*, show the same abscesses as those described by Green and Mellanby. As the doses of cod-liver oil given to different groups of rats increase, the proportion of rats which die diminishes, the proportion which resume growth increases, and also the mean increases of the groups are greater. Thus the first effect of the addition of the smallest doses of cod-liver oil to groups of rats may be described as the anti-infective effect, by which the proportion which die in 5 weeks is steadily reduced. We see that as the dose increases, this effect passes, by insensible gradations, into the growth-promoting action, and we conclude that, for purposes of measurement at any rate, the anti-infective property and the growth-promoting property are merely different aspects of the same thing.

The third point of discussion arises from the finding by Irwin, Brandt and Nelson [1930, 2] that, in their experiments, of the three factors, total food eaten, initial weight and experimental period, the total food eaten has by far the greatest influence on the gain in weight of the animals. They suggest, therefore, that some technique for the regulation of the food intake of the animals would lead to less variability in response. We have not made records of the daily intake of basal diet in these experiments, but it would appear to be unnecessary for our purpose to do so, as we have obtained comparable results on small numbers of animals without considering this factor.

SUMMARY.

A method is described by which the vitamin A content of two substances may be compared, using two groups of rats, there being five or preferably ten rats in each group.

Each group of rats is fed on vitamin A-free diet until growth ceases and each rat within the group is then given the same daily dose of the substance to be tested. The mean increases in weight of the two groups are calculated.

These mean increases are then referred to a curve which relates mean increase to dose of a particular sample of cod-liver oil. The relative potency of the two substances is calculated from the doses of cod-liver oil corresponding to the mean increases of the groups used in testing these substances.

When 10 rats are used in a group, the potency of a substance can usually be estimated with an error of less than 30 %.

The curve relating dose to mean increase of weight in a group cannot be assumed to be applicable to all vitamin A-free diets. It was found, indeed, that the use of one type of caseinogen made it impossible to construct such a curve at all.

The writers would express their warm thanks to Dr J. H. Burn for his generous help and advice in this work.

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